Chemical Analysis and Testing
Laboratory Analytical Procedures

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(as of 09-23-98)

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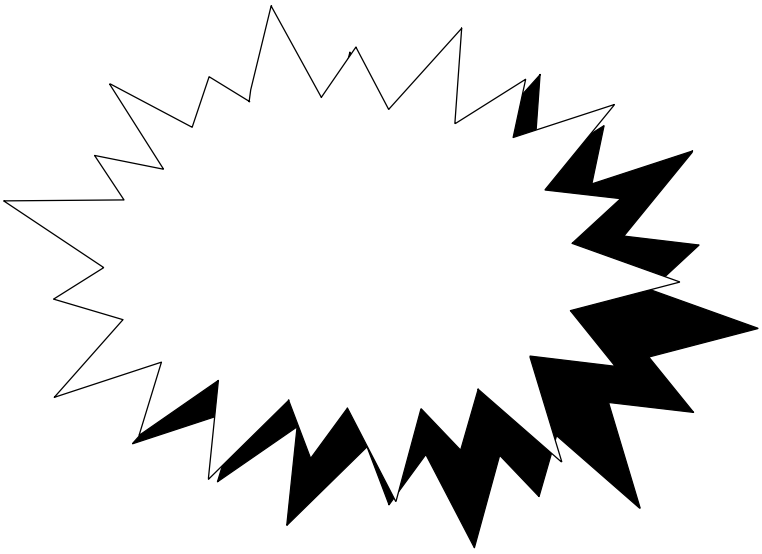

Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-001

Procedure Title: Standard Method for Determination of Total Solids in Biomass

Author: Tina Ehrman

Date: 10/28/94

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Standard Method for Determination of Total Solids in Biomass

Laboratory Analytical Procedure #001

1. Introduction

1.1 Biomass samples are hygroscopic materials and can contain large and varying amounts of moisture. To be meaningful, the results of chemical analyses of biomass are typically reported on a dry weight basis. The following procedure describes the method used to determine the amount of solids (or moisture) present in a solid biomass sample.

1.2 This procedure has been adopted by ASTM as an ASTM Standard Test Method.

2. Scope

2.1 This method is intended to determine the amount of total solids remaining after 105^{o}C drying of a solid sample.

2.2 All analyses shall be performed according to the guidelines established in the Ethanol Project Quality Assurance Plan (QAP).

3. References

3.1 NREL CAT Task Laboratory Analytical Procedure #012, "Standard Test Method for Moisture, Total Solids, and Total Dissolved Solids in Biomass Slurry and Liquid Process Samples."

3.2 TAPPI Method T210 om-58. 1991. "Weighing, Sampling and Testing Pulp for Moisture." Technical Association of the Pulp and Paper Industry Standard Methods.

3.3 Vinzant, T.B., L. Ponfick, N.J. Nagle, C.I. Ehrman, J.B. Reynolds, and M.E. Himmel. 1994. "SSF Comparison of Selected Woods From Southern Sawmills." Appl. Biochem. Biotechnol. 45/46:611-626.

4. Significance and Use

4.1 The total solids content of a biomass sample is the amount of solids remaining after all volatile matter has been removed by heating the sample at 105EC to constant weight. Conversely, the moisture content is a measure of the amount of water (and other components volatilized at 105^{o}C) present in such a sample.

4.2 The results of the chemical analyses of biomass samples are typically reported on a 105°C dry weight basis. The total solids content of a sample is used to convert the analytical results obtained on another basis to that of a dry weight basis.

5. Apparatus

5.1 Analytical balance, sensitive to 0.1 mg.

5.2 Automated infrared moisture analyzer (such as Denver Instrument Company IR-100 or equivalent) or a convection (drying) oven, with temperature control of 105 ± 3°C.

5.3 Desiccator.

5.4 Aluminum foil weighing dishes.

6. ES&H Considerations and Hazards

6.1 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

7. Sampling, Test Specimens and Test Units

7.1 Test specimens suitable for analysis by this procedure are "as received", air-dried, milled, or extractive-free biomass solids and the solid fraction of samples generated during the pretreatment, fractionation, or fermentation of biomass. If the total solids (or moisture) content of the whole slurry or liquid fraction of these process samples are to be determined, the CAT Task Laboratory Analytical Procedure #012, "Standard Test Method for Moisture, Total Solids, and Total Dissolved Solids in Biomass Slurry and Liquid Process Samples", must be used instead.

7.2 The test specimen shall consist of approximately 2 to 10 g of sample obtained in such a manner as to ensure that it is representative of the entire lot of material being tested.

7.3 Materials containing a large amount of free sugars or proteins will caramelize or brown under direct infrared heating elements. Total solids in these materials should be done by the Convection Oven Procedure.

7.4 This procedure is not suitable for biomass samples that visibly change on heating, such as unwashed acid-pretreated biomass still containing free acid.

8. Convection Oven Procedure

8.1 This method involves drying a sample at 105°C ± 3°C in a convection oven. Each sample must be run in replicate (duplicates, at minimum).

8.2 Accurately weigh a predried aluminum foil weighing dish to the nearest 0.1 mg. Record this weight.

8.3 Thoroughly mix the sample and then weigh out 1 to 5 grams, to the nearest 0.1 mg, into the weighing dish. Record the weight of the sample plus weighing dish.

8.4 Place the sample into a convection oven at 105°C ± 3°C and dry to constant weight (±0.1% change in the amount of moisture present upon one hour of reheating). Typically overnight drying is required for very wet samples.

8.5 Remove the sample from the oven and place in a desiccator; cool to room temperature.

8.6 Weigh the dish containing the oven-dried sample to the nearest 0.1 mg and record this weight.

9. Infrared Moisture Analyzer Procedure

9.1 This method employs an automated infrared moisture analyzer. Each sample should be run in replicate (duplicates, at minimum).

9.2 Program the automated moisture analyzer for a standby temperature of 95^{o}C, an analysis temperature of 105^{o}C, and a pre-determined end of analysis criteria of a rate of weight change that does not exceed 0.05% in one minute.

9.3 Turn on the infrared heating elements. Once the analysis temperature of 105^{o}C has been reached, allow the instrument to equilibrate at that temperature for 30 minutes.

9.4 Place an aluminum foil weighing dish on the balance pan. For wetter samples, it may be useful to place a quartz pad in the weighing dish to help disperse the moisture. Shut the hood of the instrument and wait five minutes to insure that the dish and pad are completely dry before taring the balance.

9.5 Quickly transfer 1 to 3 grams of the thoroughly mixed sample to the weighing dish. Spread the sample as evenly as possible over the surface of the weighing dish.

9.6 As soon as the instrument balance stabilizes, shut the hood of the instrument and proceed with the analysis, following the instructions in the instrument operation manual.

9.7 Once the sample has been dried to constant weight, as determined by the programmed analysis parameters, the analysis will be automatically terminated by the instrument.

10. Calculations

10.1 Calculate the percent total solids on a 105°C dry weight basis as follows (the automated moisture analyzer will provide the calculated value as part of the instrument printout):

$$\%\ Total\ \ solids\ = \frac{weight\ \ dried\ \ sample\ \ plus\ \ dish\ -\ weight\ \ dish}{weight\ \ sample\ \ as\ received}\ x\ 100$$

10.2 If desired, the percent moisture can also be calculated:

$$\%\ Moisture = [1 - \frac{(weight\ dried\ sample\ plus\ dish\ -\ weight\ dish)}{weight\ sample\ as\ received}]\ x\ 100$$

11. Report

11.1 Report the result as percent total solids (or percent moisture) to two decimal places, and cite the basis used in the calculations.

11.2 For replicate analyses of the same sample, report the average, standard deviation, and %RPD.

12. Precision and Bias

12.1 Convection Oven Procedure: Data obtained by replicate testing of hybrid poplar in one laboratory gave a standard deviation in moisture content of 0.19% and a CV% of 0.20%. Replicate testing of sodium tartrate gave a standard deviation in total solids of 0.21% and a CV% of 0.23%.

12.2 Infrared Moisture Analyzer Procedure: Data obtained by replicate testing of hybrid poplar in one laboratory gave a standard deviation in moisture content of 0.20% and a CV% of 0.21%. Replicate testing of sodium tartrate gave a standard deviation in total solids of 0.58% and a CV of 0.60%.

12.3 An inherent error in any moisture determination involving drying of the sample is that volatile substances other than water are removed from the sample during drying.

13. Quality Control

13.1 *Reported significant figures:* Report the percent total solids (or percent moisture) to two decimal places.

13.2 *Replicates:* At minimum, all samples and the method verification standard are to be analyzed in duplicate.

13.3 *Blank:* This gravimetric analysis utilizes a balance blank with every batch of samples, consisting of a weighing dish passed through all steps of the procedure. The difference in weight must be less than the equivalent of a 0.5% error.

13.4 *Relative percent difference criteria:* For the infrared drying method the maximum %RPD for duplicate analysis of a sample is 4.0%. For the convection oven method the maximum %RPD is 1.1%. If the stated %RPD is exceeded, the sample must be rerun.

13.5 *Method verification standard:* A method verification standard must be run in duplicate with every batch. Sodium tartrate is a suitable material for use as a method verification standard, since the moisture content of this material is not greatly affected by its storage conditions. The published "loss on drying" for sodium tartrate is 15.62% (84.38% total solids).

13.6 *Calibration verification standard:* Not applicable.

13.7 *Sample size:* A minimum of two grams of sample are required for duplicate analyses. If there is insufficient sample, the result will be flagged and the lack of precision will be noted.

13.8 *Sample storage:* Samples shall be stored in an airtight container. Process samples and high-moisture-content feedstock samples must be refrigerated. Every effort shall be made to ensure that a representative aliquot is taken for analysis.

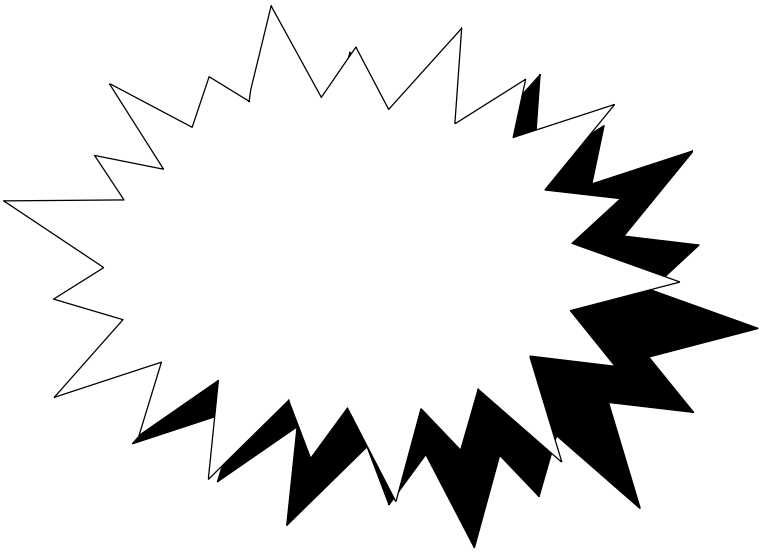
13.9 *Standard storage:* Not applicable.

13.10 *Standard preparation:* Not applicable.

13.11 *Definition of a batch:* Any number of samples which are analyzed and recorded together. The maximum size of a batch would be limited by the equipment constraints. A batch cannot be larger than what is practical with the equipment.

13.12 *Control charts:* The result of each replicate analysis of the method verification standard is recorded along with the average, %RPD, and a laboratory book/page reference. The average value obtained for each analysis of the method verification standards is to be control charted.

13.13 *Other:* Biomass can rapidly gain or lose moisture when in contact with air. During the weighing steps, minimize the amount of time the sample is exposed to the air.



Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-002

Procedure Title: **Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography**

Author: Raymond Ruiz and Tina Ehrman

Date: **2-14-96**

ISSUE DATE: **8-12-96** **SUPERSEDES:** **1-30-95**

Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography

Laboratory Analytical Procedure #002

1. Introduction

1.1 The carbohydrates making up a major portion of biomass samples are polysaccharides composed primarily of glucose, xylose, arabinose, galactose, and mannose subunits. The polysaccharides present in a biomass sample can be hydrolyzed to their component sugar monomers by sulfuric acid in a two-stage hydrolysis process. The sample can then be quantified by ion-moderated partition HPLC.

1.2 This procedure has been adopted by ASTM as the Standard Test Method for Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography, E1758-95.

2. Scope

2.1 This method covers the determination of carbohydrates, expressed as the percent of each sugar present in a hydrolyzed biomass sample. The sample is taken through a primary 72% sulfuric acid hydrolysis, followed by a secondary dilute-acid hydrolysis.

2.2 Sample material suitable for this procedure include hard and soft woods, herbaceous materials (such as switchgrass and sericea), agricultural residues (such as corn stover, wheat straw, and bagasse), waste-paper (such as office waste, boxboard, and newsprint), washed acid- and alkaline-pretreated biomass, and the solid fraction of fermentation residues. All results are reported relative to the 105°C oven-dried weight of the sample. In the case of extracted materials, the results may also be reported on an extractives-free basis.

2.3 All analyses shall be performed according to the guidelines established in the Ethanol Project Quality Assurance Plan (QAP).

3. References

3.1 Moore, W.E., and D.B. Johnson. 1967. *Procedures for the Chemical Analysis of Wood and Wood Products.* Madison, WI: U.S. Forest Products Laboratory, U.S. Department of Agriculture.

3.2 Ethanol Project Laboratory Analytical Procedure #001, "Standard Method for the Determination of Total Solids in Biomass".

3.3 Ethanol Project Laboratory Analytical Procedure #003, "Determination of Acid-Insoluble Lignin in Biomass".

3.4 NREL Ethanol Project Laboratory Analytical Procedure #004, "Determination of Acid-Soluble Lignin in Biomass".

3.5 NREL Ethanol Project Laboratory Analytical Procedure #010, "Standard Method for the Determination of Extractives in Biomass".

3.6 TAPPI Test Method T264 om-88, "Preparation of Wood For Chemical Analysis." *In Tappi Test Methods.* Atlanta, GA: Technical Association of the Pulp and Paper Industry.

3.7 Vinzant, T.B., L. Ponfick, N.J. Nagle, C.I. Ehrman, J.B. Reynolds, and M.E. Himmel. 1994. "SSF Comparison of Selected Woods From Southern Sawmills." Appl. Biochem. Biotechnol., 45/46:611-626.

4. Terminology

4.1 Prepared Biomass - Biomass that has been prepared by lyophilization, oven drying, air drying, and in some instances by extraction, to reduce the moisture content of the sample so it is suitable for carbohydrate analysis.

4.2 Oven-Dried Weight - The moisture-free weight of a biomass sample as determined by LAP-001, "Standard Method for Determination of Total Solids in Biomass".

5. Significance and Use

5.1 The percent sugar content is used in conjunction with other assays to determine the total composition of biomass samples.

6. Interferences

6.1 Samples with high protein content may result in percent sugar values biased low, as a consequence of protein binding with some of the monosaccharides.

6.2 Test specimens not suitable for analysis by this procedure include acid- and alkaline-pretreated biomass samples that have not been washed. Unwashed pretreated biomass samples containing free acid or alkali may change visibly on heating.

7. Apparatus

7.1 Hewlett Packard Model 1090 HPLC, or equivalent, with refractive index detector.

7.2 HPLC columns, BioRad Aminex7 HPX-87C and/or Aminex7 HPX-87P (or equivalent).

7.3 Guard columns, cartridges appropriate for the column used.

> Note: Deashing guard column cartridges from BioRad, of the ionic form $H^+/CO_3^{\%}$, are an option when using an HPX-87P column. These cartridges have been found to be effective in eliminating baseline ramping.

7.4 Analytical balance readable to 0.1 mg.

7.5 Convection ovens with temperature control to 45 ± 3°C and 105 ± 3°C.

7.6 Autoclave capable of maintaining 121 ± 3°C.

7.7 Water bath set at 30 ± 3°C.

7.8 Desiccator containing anhydrous calcium sulfate.

8. Reagents and Materials

8.1 Reagents

8.1.1 High purity sugars for standards (98%+) - two sets of glucose, xylose, galactose, arabinose,and mannose from different lots or manufacturers.

8.1.2 72% w/w H_2SO_4 (12.00 ± 0.02 M or specific gravity 1.6389 at 15.6 °C /15.6°C).

8.1.3 Calcium carbonate, ACS reagent grade.

8.1.4 Water, 18 megohm deionized.

8.2 Materials

8.2.1 Glass test tubes, 16x100 mm.

8.2.2 125 mL glass serum bottles, crimp top style, with rubber stoppers and aluminum seals to fit.

8.2.3 pH paper, suitable to cover the pH range of 4 to 7.

8.2.4 Disposable nylon syringe filters, 0.2 μm.

8.2.5 Disposable syringes, 3 mL.

8.2.6 Autosampler vials, with crimp top seals to fit.

8.2.7 Erlenmeyer flasks, 50 mL.

9. ES&H Considerations and Hazards

9.1 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

9.2 72% H_2SO_4 is very corrosive and must be handled carefully.

9.3 Use caution when handling hot glass bottles after the autoclave step, as they may have become pressurized.

10. Sampling, Test Specimens and Test Units

10.1 Test specimens suitable for analysis by this procedure are as follows:

- biomass feedstocks, dried and reduced in particle size, if necessary.
- pretreated biomass, washed free of any residual acid or alkali.
- the solids fraction of fermentation residues.

10.2 The sample must not contain particles larger than 1 mm in diameter. If milling is required to reduce the particle size of the test specimen, a laboratory mill equipped with a 40 mesh (or smaller) screen should be used.

10.3 The total solids content of the "as received" test specimen (prior to any drying or extraction steps) must be determined by LAP-001 in parallel with the carbohydrate analysis. Record this value as $\%T_{as\ received}$.

10.4 Material with a total solids content less than 85%, on a 105°C dry weight basis, will require drying by lyophilization, oven drying, or air drying prior to milling or analysis. The amount of moisture lost as a result of the preparation procedure must be determined. This moisture content is used to calculate the total solids content of the sample based on its preparation and is recorded as $\%T_{prep}$. This value is used to correct the weight of the prepped material used in the carbohydrate analysis, as described in the calculations section. The prepared sample should be stored in a manner to ensure its moisture content does not change prior to analysis.

> Note: Preparing samples for analysis by oven drying can produce hard chunks of material. This material must then be milled to reduce the size of the large pieces to less then 1 mm in diameter. The sample is then redried prior to testing.

10.5 Some samples may require extraction prior to analysis, to remove components that may interfere with the analysis. LAP-010, "Standard Method for the Determination of Extractives in Biomass", is used to prepare an extractives-free sample with a moisture content suitable for carbohydrate analysis. As part of this procedure, the percent extractives in the prepared sample, on a 105°C dry weight basis, is determined. This value, recorded as % extractives, can be used to convert the % sugar reported on a extractives-free basis to an as received (whole sample) basis.

10.6 The test specimen shall consist of approximately 0.3 g of sample. The test specimen shall be obtained in such a manner to ensure that it is representative of the entire lot of material being tested.

11. Procedure

11.1 This procedure is suitable for air-dried, lyophilized, and extracted biomass samples, as well as for samples that have been oven dried at a temperature of 45°C or less. It is not suitable for samples that have been dried at a temperature exceeding 45°C.

Note: The total solids content of the original sample, $\%T_{as\ received}$, must be determined using LAP-001, prior to any preparatory steps. The total solids content of the sample based on its preparation, $\%T_{prep}$, must also be known.

11.2 Determine the total solids content of the prepared or extractives-free biomass sample by LAP-001 and record this value as $\%T_{final}$.

Note: Samples for total solids determination (LAP-001) must be weighed out at the same time as the samples for the carbohydrate determination. If this is done later, it can introduce an error in the calculation because ground biomass can rapidly gain or lose moisture when exposed to the atmosphere.

11.3 Weigh 0.3 ± 0.01 g of the prepared or extractives-free sample to the nearest 0.1 mg and place in a 16x100 mm test tube. Record as W_1, the initial sample weight in grams. Each sample must be run in duplicate, at minimum.

11.4 Add 3.00 ± 0.01 mL (4.92 ± 0.01 g) of 72% H_2SO_4 and use a glass stirring rod to mix for 1 minute, or until the sample is thoroughly wetted.

11.5 Place the test tube in the water bath set at 30 ± 1°C and hydrolyze for 2 hours.

11.6 Stir the sample every 15 minutes to assure complete mixing and wetting.

11.7 Weigh out 0.3 ± 0.01 g of each high purity sugar (predried at 45°C) to the nearest 0.1 mg, and place each in its own 16x100 mm glass test tube. Add acid, hydrolyze, and stir these sugars as described in the previous three steps. These sugar recovery standards (SRS) will be taken through the remaining steps in the procedure in parallel with the samples. The calculated recovery of the SRSs will be used to correct for losses due to the destruction of sugars during the hydrolysis process. It may be useful to run selected SRSs in duplicate, particularly if specific sugars are deemed critical.

11.8 Prepare a method verification standard (MVS) by weighing out 0.3 ± 0.01 g of a well characterized standard material suitable for analysis. Add acid, hydrolyze, and stir the MVS as was done with the samples and SRSs (see 11.4-11.6 above). This MVS will be taken through the remaining steps in the procedure in parallel with the samples and the SRSs, and is used to test the reproducibility of the method as a whole.

Note: A suitable method verification standard, *Populus deltoides*, may be obtained from NIST (research material #8492).

11.9 Upon completion of the two hour hydrolysis step, transfer each hydrolyzate to its own serum bottle and dilute to a 4% acid concentration by adding 84.00 ± 0.04 mL deionized water. Be careful to transfer all residual solids along with the hydrolysis liquor. The total weight added to the tared bottle is 89.22 g (0.3 g sample, 4.92 g 72% H_2SO_4, and 84.00 g deionized water). Since the specific gravity of the 4% acid solution is 1.0250 g/mL, the total volume of solution, V_F, is 87.0 mL.

11.10 Stopper each of the bottles and crimp aluminum seals into place.

11.11 Set the autoclave to a liquid cycle to prevent loss of sample from the bottle in the event of a loose crimp seal. Autoclave the samples in their sealed bottles for 1 hour at 121 ± 3°C.

11.12 After completion of the autoclave cycle, allow the samples to cool for about 20 minutes at room temperature before removing the seals and stoppers.

11.13 These autoclaved solutions may also be used for the determination of acid-insoluble residue and/or acid-soluble lignin, in parallel with this carbohydrate determination.

> Note: If acid-insoluble lignin and/or acid-soluble lignin determinations are to be conducted on a sample, the residual solids must be collected by filtering the hydrolyzate through an ashed and weighed filtering crucible prior to proceeding with the carbohydrate determination. Refer to LAP-003, "Determination of Acid-Insoluble Lignin in Biomass", for details. If an acid-soluble lignin determination is to be conducted, a portion of the filtrate must be reserved for analysis. Acid-soluble lignin should be analyzed within 24 hours, preferably within 6 hours of hydrolysis. Refer to the procedure "Determination of Acid-Soluble Lignin in Biomass" (LAP-004) for details.

11.14 Transfer 20 mL aliquots of each hydrolyzate, or filtrate, to 50 mL Erlenmeyer flasks.

11.15 Neutralize with calcium carbonate to a pH between 5 and 6. Do not over-neutralize. Add the calcium carbonate slowly with frequent swirling to avoid problems with foaming. Monitor the pH of the solution with pH paper to avoid over-neutralization.

11.16 Filter the neutralized hydrolyzate using a 3 mL syringe with a 0.2 µm filter attached. One portion of the hydrolyzate should be filtered directly into a sealable test tube for storage. A second portion should be filtered directly into an autosampler vial if the hydrolyzate is to be analyzed without dilution. If the concentration of any of the analytes is expected to exceed the validated linear range of the analysis, dilute the hydrolyzate as required and filter into an autosampler vial for analysis.

> Note: It is advisable to determine the initial glucose concentration of the sample using an alternative technique, such as a YSI glucose analyzer, in order to predict whether or not the glucose in the sample will fall within the linear range of the analysis.

11.17 The portion of the neutralized hydrolyzate filtered into the test tube should be securely sealed, labeled, placed in the refrigerator, and reserved in case a repeat analysis is required. The sample should be stored for no longer than two weeks.

11.18 Prepare a series of sugar calibration standards in deionized water at concentrations appropriate

for creating a calibration curve for each sugar of interest. A suggested scheme for the HPX-87C column is to prepare a set of multi-component standards containing glucose, xylose, and arabinose in the range of 0.2 -12.0 mg/mL. For the HPX-87P column, galactose, and mannose should be included as additional components in the standards. Extending the range of the calibration curves beyond 12.0 mg/mL will require validation.

11.19 Prepare an independent calibration verification standard (CVS) for each set of calibration standards, using sugars obtained from a source other than that used in preparing the calibration standards. The CVS must contain precisely known amounts of each sugar contained in the calibration standards, at a concentration that falls in the middle of the validated range of the calibration curve. The CVS is to be analyzed after each calibration curve and at regular intervals in the HPLC sequence, bracketing groups of samples. The CVS is used to verify the quality of the calibration curve(s) throughout the HPLC run.

11.20 Analyze the calibration standards, the CVS, the samples, the SRSs, and the MVS by HPLC using a Biorad Aminex7 HPX-87C or HPX-87P column for glucose, xylose, and arabinose. If mannose and galactose are also to be determined, a Biorad Aminex7 HPX-87P column must be used instead. For many analyses, it is useful to run the same samples on both columns and compare the results. The following instrumental conditions are used for both the HPX-87C and the HPX-87P columns:

Sample volume: 50 µL.
Eluant: 0.2 µm filtered and degassed, deionized water.
Flow rate: 0.6 mL/min.
Column temperature: 85°C.
Detector: refractive index.
Run time: 20 minutes data collection plus a 15 minute post-run.

12. Calculations

12.1 Create a calibration curve by linear regression analysis for each sugar to be quantified. From these curves, determine the concentration in mg/mL of the sugars present in each solution analyzed by HPLC.

12.2 Calculate the amount of sugar recovered from each SRS taken through the two-stage hydrolysis. This will give an estimate of the amount of each individual sugar destroyed during the hydrolysis procedure.

$$\% R_{srs} = \frac{C_2}{C_1} x\, 100\%$$

Where: $\%R_{srs}$ = % recovery of sugar recovery standard
C_1 = known concentration of sugar recovery standard before hydrolysis, in mg/mL
C_2 = concentration of sugar recovery standard detected by HPLC after hydrolysis, in mg/mL

12.3 Use the percent recovery of the appropriate sugar recovery standard to correct sugar concentration values (in mg/mL) obtained from HPLC for each sugar detected in the hydrolyzed sample.

$$C_{corr} = C_{spl} \div \frac{\%\, R_{srs}}{100\%}$$

Where:
C_{corr} = concentration of sugar in hydrolyzed sample corrected for hydrolysis, in mg/mL
C_{spl} = concentration of sugar detected in the hydrolyzed sample by HPLC, in mg/mL
$\%R_{srs}$ = % recovery of sugar recovery of standard, as determined in the previous step

12.4 For lyophilized, air dried, or oven dried samples, or for samples requiring no preparation, calculate the percentage of each sugar present in the sample on an as received 105°C dry weight basis as follows:

$$\%\ Sugar = \frac{C_{corr}\ x\ \frac{1\ g}{1000\ mg}\ x\ V_F}{W_1\ x\ \frac{\%\, T_{as\ received}}{\%\, T_{prep}}}\ x\ 100\% = \frac{C_{corr}\ x\ \frac{1\ g}{1000\ mg}\ x\ V_F}{W_1\ x\ \frac{\%\, T_{final}}{100\%}}\ x\ 100\%$$

Where: W_1 = initial weight of sample, in grams
V_F = volume of filtrate, 87.0 mL
C_{corr} = concentration of sugar in hydrolyzed sample corrected for loss on hydrolysis, is determined in previous step, in mg/mL
$\%T_{as\ received}$ = % total solids content of the original sample (prior to any preparation steps) on a 105°C dry weight basis, as determined by the LAP-001
$\%T_{prep}$ = % total solids content of the sample as determined during the preparation of the sample for analysis (by lyophilization, oven-drying, or air drying)
$\%T_{final}$ = % total solids content of the prepared sample used in this carbohydrate analysis, on a 105°C dry weight basis, as determined by the LAP-001

Note: If the sample used in the analysis required no preparation (analyzed as received), then $\%T_{prep} = 100\%$ and $\%T_{final} = \%T_{as\ received}$.

12.5 If the biomass was prepared according to the "Standard Method for the Determination of Ethanol Extractives in Biomass" (LAP-010), first calculate the percentage of each sugar present on an extractives-free 105EC dry weight basis and then correct this value to an as received (whole sample) 105°C dry weight basis.

12.5.1 Calculate the percentage of each sugar on an extractives-free basis as follows:

$$\%\ Sugar_{extractives\text{-}free} = \frac{C_{corr} \times \frac{1\ g}{1000\ mg} \times V_F}{W_1 \times \frac{\%\ T_{final}}{100\%}} \times 100\%$$

Where:
C_{corr} = concentration of sugar in hydrolyzed sample corrected for loss on hydrolysis, as determined above, in mg/mL
V_F = volume of filtrate, 87.0 mL
W_1 = initial weight of extracted sample, in grams
$\%T_{final}$ = % total solids content of the prepared sample used in this carbohydrate analysis (in this case, extracted sample), on a 105°C dry weight basis, as determined by the LAP-001

12.5.2 Correct the % sugar value on an extractives-free basis, calculated above, to an as received (whole sample) 105°C dry weight basis as follows:

$$\%\ Sugar_{whole\ sample} = \%\ Sugar_{extractives\text{-}free} \times \frac{(100\% - \%\ extractives)}{100\%}$$

Where:
% Sugar$_{extractives\text{-}free}$ = % sugar on an extractives-free 105°C dry weight basis, as determined in the previous step
% extractives = % extractives in the extracted sample as described in the Standard Method for the Determination of Extractives in Biomass

13. Report

13.1 Report the percent sugar present in the sample, to two decimal places, on a whole sample 105°C dry weight basis or on an extractives-free basis. Cite the basis used in the report.

13.2 For replicate analyses of the same sample, report the average, standard deviation, and relative percent difference (RPD).

14. Precision and Bias

14.1 Data obtained by replicate testing of a hybrid poplar in one laboratory, using a HPX-87P column, gave a standard deviation in glucose content of 1.90% and a CV of 3.95%.

14.2 Data obtained by replicate testing of an extractives-free hybrid poplar sample in five different laboratories gave a standard deviation of 1.90% and a CV of 4.0%.

15. Quality Control

15.1 *Reported significant figures:* Report the percentage of each sugar present in the hydrolyzed sample to two decimal places, on a whole sample 105°C dry weight basis or on an extractives-free basis. Cite the basis used in the calculation.

15.2 *Replicates:* At minimum, all samples and the method verification standard are to be analyzed in duplicate.

15.3 *Blank:* The only requirement is a reagent blank, which starts out as an empty 16x100 mm test tube (ie, no sample) which is taken through all the procedural steps.

15.4 *Relative percent difference criteria:* The RPD for glucose must be less than 6.1%. If the RPD is too large, the sample must rerun.

15.5 *Method verification standard:* A method verification standard must be run in duplicate with every batch. This method utilizes a well characterized standard material suitable for analysis. For example, NIST 8492 (*Populus deltoides*) is used as the MVS in carbohydrate analysis of hardwoods.

15.6 *Calibration verification standard:* Calibration verification standards shall be independently prepared and analyzed as described in section 11.19 of this procedure.

15.7 *Sample size:* A minimum of 0.6 grams of sample (on a dry weight basis) are required for duplicate analyses. If there is insufficient sample, the result will be flagged and the lack of precision will be noted.

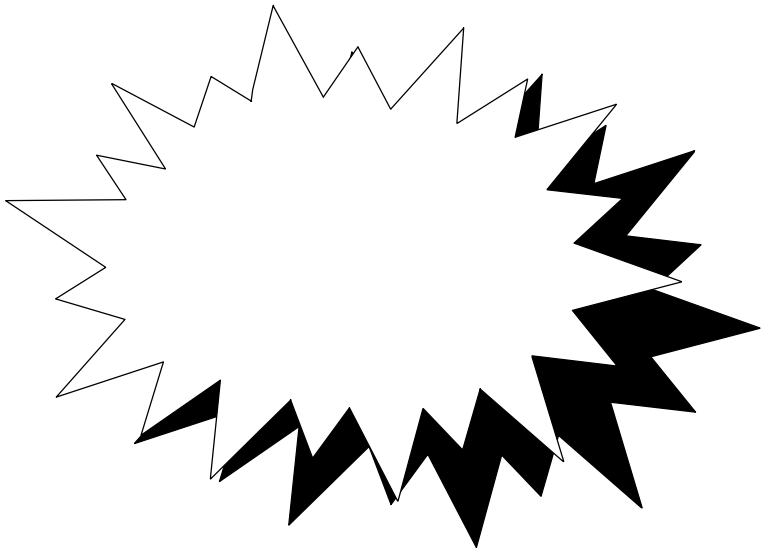
15.8 *Sample storage:* Samples shall be stored in an airtight container and refrigerated.

15.9 *Standard storage:* Standards should be kept frozen in airtight vials or test tubes. Vortex the standards vigorously upon thawing to ensure thorough mixing.

15.10 *Standard preparation:* Standards are prepared according to section 11.18 of this procedure.

15.11 *Definition of a batch:* Any number of samples which are analyzed and recorded together. The maximum size of a batch would be limited by the equipment constraints. A batch cannot be larger than what is practical with the equipment.

15.12 *Control charts:* The result of each replicate analysis of the method verification standard is recorded along with the average, RPD, and a laboratory book/page reference. The average value obtained for each analysis of the method verification standards is to be control charted.



Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-003

Procedure Title: **Determination of Acid-Insoluble Lignin in Biomass**

Author: David Templeton and Tina Ehrman

Date: **1-19-95**

ISSUE DATE: **1-30-95**

SUPERSEDES: 8/19/92

Determination of Acid-Insoluble Lignin in Biomass

Laboratory Analytical Procedure #003

1. Introduction

1.1 Biomass is composed largely of cellulose, a polymer of glucose; hemicellulose, a complex polymer of which the main chain consists primarily of xylans or glucomannans; and lignin, a complex phenolic polymer. Unlike the other cell wall components of biomass, the lignin is mostly insoluble in mineral acids. For this reason, lignin can be analyzed gravimetrically after hydrolyzing the cellulose and hemicellulose fractions with sulfuric acid.

1.2 This method contains two different procedures for determining acid-insoluble lignin. Both approaches have been shown to give equivalent results. Procedure A presents an approach where the acid-insoluble lignin procedure also generates the solutions required for total carbohydrate and acid-soluble lignin determinations, thereby making possible the "summative" analysis of the same sample. Procedure B is a modification of the classic "Klason lignin" determination. Although the filtrate generated from this procedure can be used to determine acid-soluble lignin, total carbohydrates should be determined on a completely separate sample.

1.3 This procedure has been adopted by ASTM as an ASTM Standard Test Method.

2. Scope

2.1 This test method covers the determination of acid-insoluble lignin of hard and soft woods, herbaceous materials (such as switchgrass and sericea), agricultural residues (such as corn stover, wheat straw, and bagasse), wastepaper (such as office waste, boxboard, and newsprint), acid and alkaline pretreated biomass, and the solid fraction of fermentation residues. All results are reported relative to the 105^{o}C oven-dried weight of the sample. In the case of extracted materials, the results may also be reported on an extractives-free basis.

2.2 The residue collected contains the acid-insoluble lignin and any condensed proteins from the original sample. An independent nitrogen analysis would be required to determine the acid-insoluble lignin content

separate from the condensed protein fraction and is outside the scope of this procedure.

2.3 All analyses shall be performed according to the guidelines established in the Ethanol Project Quality Assurance Plan (QAP).

3. References

3.1 Ehrman, C.I., and M.E. Himmel. 1994. "Simultaneous Saccharification and Fermentation of Pretreated Biomass: Improving Mass Balance Closure." *Biotechnology Techniques*, 8(2):99-104.

3.2 Moore, W.E., and D.B. Johnson. 1967. *Procedures for the Chemical Analysis of Wood and Wood Products.* Madison, WI: U.S. Forest Products Laboratory, U.S. Department of Agriculture.

3.3 NREL CAT Task Laboratory Analytical Procedure #001, "Standard Method for the Determination of Total Solids in Biomass".

3.4 NREL CAT Task Laboratory Analytical Procedure #002, "Two Stage Sulfuric Acid Hydrolysis for Determination of Carbohydrates".

3.5 NREL CAT Task Laboratory Analytical Procedure #004, "Determination of Acid-Soluble Lignin in Biomass".

3.6 NREL CAT Task Laboratory Analytical Procedure #010, "Standard Method for the Determination of Extractives in Biomass".

3.7 TAPPI Test Method T222 om-88, "Acid-Insoluble Lignin in Wood and Pulp." *In Tappi Test Methods.* Atlanta, GA: Technical Association of the Pulp and Paper Industry.

3.8 TAPPI Test Method T264 om-88, "Preparation of Wood For Chemical Analysis." *In Tappi Test Methods.* Atlanta, GA: Technical Association of the Pulp and Paper Industry.

3.9 Vinzant, T.B., L. Ponfick, N.J. Nagle, C.I. Ehrman, J.B. Reynolds, and M.E. Himmel. 1994. "SSF Comparison of Selected Woods From Southern Sawmills." Appl. Biochem. Biotechnol., 45/46:611-626.

4. Terminology

3.10 Acid-insoluble lignin is defined to be the residue, corrected for acid-insoluble ash, retained on a medium porosity filter crucible after the primary 72% and secondary 4% H_2SO_4 hydrolysis steps described in this procedure.

5. Significance and Use

3.11 The acid-insoluble lignin content is used in conjunction with other assays to determine the total composition of biomass samples.

6. Interferences

3.12 The results of acid-insoluble lignin analysis are affected by incomplete hydrolysis of biomass. Unless the sample is hydrolyzed completely, the results will be biased high. Take care to mix the acid/biomass slurry thoroughly at the beginning and periodically throughout the concentrated acid hydrolysis.

3.13 The results of acid-insoluble lignin analysis are affected by the timing of the acid digestion steps. The insoluble lignin will slowly dissolve into solution in an irreproducible fashion. The timing within this procedure must be followed closely.

3.14 Some proteinaceous materials can also form acid-insoluble substances that are collected with acid-insoluble lignin.

7. Apparatus

3.15 Analytical balance readable to 0.1 mg.

3.16 Convection oven with temperature control of $105 \pm 3^{o}C$.

3.17 Muffle furnace: an electric furnace is recommended for igniting the sample. The furnace should be fitted with an indicating pyrometer or thermocouple, so that the required temperature of $575 \pm 25^{o}C$ can be maintained.

3.18 Autoclave capable of maintaining $121 \pm 3^{o}C$ (Procedure A) or heating manifold equipped with reflux condensers with 24/40 ground glass joints (Procedure B).

3.19 Water bath set at $30 \pm 1^{o}C$ (Procedure A).

3.20 Filtration set-up including vacuum source and vacuum adapters for crucibles.

3.21 Desiccator containing anhydrous calcium sulfate.

8. Reagents and Materials

3.22 Reagents

3.22.1 72% w/w H_2SO_4 (12.00 ± 0.02 M or specific gravity 1.6389 at $15.6^{o}C/15.6^{o}C$).

3.22.2 Water, 18 megohm deionized.

3.23 Materials

3.23.1 Glass test tubes, 16x100 mm (Procedure A) or 20x150 mm (ProcedureB).

3.23.2 125 mL glass serum bottles, crimp top style, with rubber stoppers and aluminum seals to fit (Procedure A).

3.23.3 Erlenmeyer flask, 1000 mL, with 24/40 ground glass joint (Procedure B).

3.23.4 Filtration flask, 250 mL (Procedure A) or 1000 mL (Procedure B).

3.23.5 30 mL (Procedure A) or 50 mL (Procedure B) glass filtering crucible, medium porosity, nominal maximum pore size of 10 μm.

9. ES&H Considerations and Hazards

3.24 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

3.25 72% H_2SO_4 is very corrosive and must be handled carefully.

3.26 Use caution when handling glass bottles after the autoclave step in Procedure A, as they may have become pressurized.

10. Sampling, Test Specimens, and Test Units

3.27 Test specimens suitable for analysis by this procedure are as follows:

- biomass feedstocks, dried and reduced in particle size if necessary,
- pretreated biomass, washed free of any residual acid or alkali,
- the solids fraction of fermentation residues.

3.28 The sample must not contain particles larger than 1 mm in diameter. If milling is required to reduce the particle size of the test specimen, a laboratory mill equipped with a 40 mesh (or smaller) screen should be used.

3.29 The total solids content of the "as received" test specimen (prior to any drying or extraction steps) must be determined by Laboratory Analytical Procedure #001, "Determination of Total Solids in Biomass", in parallel with the lignin analysis. Record this value as $T_{as\ received}$.

3.30 Material with a total solids content less than 85%, on a 105°C dry weight basis, will require drying by lyophilization, oven drying, or air drying prior to milling or analysis. The amount of moisture lost as a result of the preparation procedure must be determined. This moisture content is used to calculate the total solids content of the sample based on its preparation and is recorded as T_{prep}. This value is used to correct the weight of the prepped material used in the lignin analysis, as described in the calculations section. The prepared sample should be stored in a manner to ensure its moisture content does not change prior to analysis.

> Note: Preparing samples for analysis by oven drying can produce hard chunks of material. This material must then be milled to reduce the size of the large pieces to less then 1 mm in diameter. The sample is then redried prior to testing.

3.31 Some samples may require extraction prior to analysis, to remove components that may interfere with the analysis. Laboratory Analytical Procedure #010, "Standard Method for the Determination of Extractives in Biomass", is used to prepare extractives-free sample with a moisture content suitable for lignin analysis. As part of this procedure, the percent extractives in the prepared sample, on a 105°C dry weight basis, is determined. This value, recorded as % extractives, is used to convert the % lignin reported on a extractives-free basis to an as received (whole sample) basis.

3.32 The test specimen shall consist of approximately 0.3 g of sample for Procedure A or approximately 1.0 g of sample for Procedure B. The test specimen shall be obtained in such a manner to ensure that it is representative of the entire lot of material being tested.

11. Procedure A - Summative Analysis

3.33 This procedure is suitable for air-dried, lyophilized, and extracted biomass samples, as well as for samples that have been oven dried at a temperature of 45°C or less. It is not suitable for samples that have been dried at a temperature exceeding 45°C.

> Note: The total solids content of the original sample, $T_{as\ received}$, as well as the total solids content determined as the sample is prepared, T_{prep}, must be known.

3.34 Individually label the crucibles needed for analysis, and ignite them at 575 " 25°C to achieve a constant weight of " 0.3 mg. Store the ignited crucibles in a desiccator until needed.

Note: In order to determine the absolute amounts of acid-insoluble residue and acid-insoluble ash, for quality control purposes, it is useful to weigh and record the ignited crucible to the nearest 0.1 mg.

3.35 Weigh 0.3 ± 0.01 g prepared sample to the nearest 0.1 mg and place in a 16x100 mm test tube. Record as W_1, the initial sample weight. Each sample must be run in duplicate, at minimum.

3.36 Samples for total solids determination (LAP-001) must be weighed out at the same time as the samples for the acid-insoluble lignin determination. If this is done later, it can introduce an error in the calculation because ground biomass can rapidly gain or lose moisture when exposed to the atmosphere. Record the average total solids value as T_{final}.

3.37 Add 3.00 ± 0.01 mL (4.92 ± 0.01 g) of 72% H_2SO_4 and use a glass stirring rod to mix for 1 minute, or until the sample is thoroughly wetted.

3.38 Place the test tube in the water bath controlled to 30 ± 1°C and hydrolyze for 2 hours.

3.39 Stir the sample every 15 minutes to assure complete mixing and wetting.

3.40 Transfer the hydrolyzate to a glass bottle and dilute to a 4% acid concentration by adding 84.00 ± 0.04 mL water, or by bringing the combined weight of sample, acid, and water up to 89.22 ± 0.04 g. Be careful to transfer all the residual solids along with the hydrolysis liquor.

3.41 Stopper each of the bottles and crimp aluminum seals into place.

3.42 Set the autoclave to a liquid vent cycle to prevent loss of sample from the bottle in the event of a loose crimp seal. Autoclave the samples in their sealed bottles for 1 hour at 121 ± 3°C.

3.43 After completion of the autoclave cycle, allow the samples to cool for about 20 minutes at room temperature before removing the seals and stoppers.

3.44 Vacuum filter the hydrolysis solution through one of the previously ignited filtering crucibles.

3.45 If a carbohydrate analysis (LAP-002) and/or an acid-soluble lignin analysis (LAP-004) is desired, decant 15-25 mL of filtrate into a resealable container. If this aliquot is not used immediately for further analysis, store in refrigerator at 4°C.

Note: Acid-soluble lignin should be analyzed within 24 hours, preferably within 6 hours of hydrolysis.

3.46 Use hot deionized water to wash any particles clinging to the glass bottle into the crucible and to wash the filtered residue free of acid using vacuum filtration.

3.47 Dry the crucible and contents at $105 \pm 3^{o}C$ for 2 hours or until constant weight is achieved ("0.3 mg upon reheating).

3.48 Cool in desiccator and record the weight, W_2, the weight of the crucible, acid-insoluble lignin, and acid-insoluble ash to the nearest 0.1 mg.

3.49 Place the crucible and contents in the muffle furnace and ignite at $575 \pm 25^{o}C$ for a minimum of 3 hours, or until all the carbon is eliminated. Heat at a rate of $10^{o}C$/min to avoid flaming. If the sample tends to flare up, the container should be partially covered during this step. Avoid heating above the maximum stated temperature. Protect the test container from strong drafts at all times to avoid mechanical loss of the test specimen.

3.50 Cool in desiccator and record the weight, W_3, the weight of the crucible and acid-insoluble ash, to the nearest 0.1 mg.

Note: The amount of acid-insoluble ash remaining in the crucible is not equal to the total ash content of the sample. Refer to Laboratory Analytical Procedure #005 if total ash is to be determined.

12. Procedure B - Klason Lignin Determination

3.51 This procedure is suitable for oven-dried samples (including those dried at temperatures between $45^{o}C$ and $105^{o}C$) as well as air-dried, lyophilized, and extracted biomass samples.

Note: The total solids content of the original sample, $T_{as\ received}$, as well as the total solids content determined as the sample is prepared, T_{prep}, must be known.

3.52 Individually label the crucibles needed for analysis, and ignite them at $575 \pm 25^{o}C$ to achieve a constant weight of $\pm$ 0.3 mg. Store the ignited crucibles in a desiccator until needed.

Note: In order to determine the absolute amounts of acid-insoluble residue and acid-insoluble ash, for quality control purposes, it is useful to weigh and record the ignited crucible to the nearest 0.1 mg.

3.53 Weigh 1.0 ± 0.05 g prepared sample to the nearest 0.1 mg and place in a 20x150 mm test tube. Record as W_1, the initial sample weight. Each sample must be run in duplicate, at minimum.

3.54 Samples for total solids determination must be weighed out at the same time as the samples for the acid-insoluble lignin determination. If this is done later, it can introduce an error in the calculation because ground biomass can rapidly gain or lose moisture when exposed to the atmosphere. Record the average total solids value as T_{final}.

3.55 Add 15.00 ± 0.02 mL of 72% H_2SO_4, chilled to 4°C in the refrigerator. Use a glass stirring rod to mix for 1 minute, or until the sample is thoroughly wetted.

3.56 Hydrolyze the sample for 2 hours at room temperature (approximately 20°C), stirring every 15 minutes to assure complete mixing and wetting.

3.57 Transfer the hydrolyzate to a 1000 mL Erlenmeyer flask and dilute to a 3% acid concentration with 560 mL of deionized water. Be careful to transfer all the residual solids along with the hydrolysis liquid.

3.58 Place the flask on the heating manifold and attach to the reflux condenser. Heat the liquid to a gentle boil. Start timing at the onset of boiling, and reflux for 4 hours ± 5 minutes.

3.59 At the end of 4 hours, rinse the condenser with a small amount of deionized water before disassembling reflux apparatus.

3.60 Vacuum filter the hydrolysis solution through one of the previously ignited filtering crucibles.

3.61 If an acid-soluble lignin determination (LAP-004) is to be run, record the weight of the collected filtrate. Decant 15-25 mL of filtrate into a resealable container. If this aliquot is not used immediately for further analysis, store in refrigerator at 4°C.

> Note: Acid-soluble lignin should be analyzed within 24 hours, preferably within 6 hours of hydrolysis.

3.62 Use hot deionized water to wash any particles clinging to the glass bottle into the crucible and to wash the filtered residue free of acid using vacuum filtration.

3.63 Dry the crucible and contents at 105 ± 3°C for 2 hours or until constant weight is achieved (±0.3 mg upon reheating).

3.64 Cool in desiccator and record the weight, W_2, the weight of the crucible, acid-insoluble lignin, and acid-insoluble ash to the nearest 0.1 mg.

3.65 Place the crucible and contents in the muffle furnace and ignite at 575 ± 25^{o}C for a minimum of 3 hours, or until all the carbon is eliminated. Heat at a rate of 10^{o}C/min to avoid flaming. If the sample tends to flare up, the container should be partially covered during this step. Avoid heating above the maximum stated temperature. Protect the test container from strong drafts at all times to avoid mechanical loss of the test specimen.

3.66 Cool in desiccator and record the weight, W_3, the weight of the crucible and acid-insoluble ash, to the nearest 0.1 mg.

Note: The amount of acid-insoluble ash remaining in the crucible is not equal to the total ash content of the sample. Refer to Laboratory Analytical Procedure #005 if total ash is to be determined.

13. Calculations

3.67 For lyophilized, air dried, or oven dried samples, or samples requiring no preparation, calculate % acid-insoluble lignin on an as received 105^{o}C dry weight basis as follows:

$$\%\ acid\text{-}insoluble\ lignin = \frac{W_2 - W_3}{W_1 \times \frac{T_{asreceived}}{T_{prep}}} \times 100\% = \frac{W_2 - W_3}{W_1 \times \frac{T_{final}}{100}\%} \times 100\%$$

Where:

W_1 = initial sample weight.
W_2 = weight of crucible, acid-insoluble lignin, and acid-insoluble ash.
W_3 = weight of crucible and acid-insoluble ash.
$T_{as\ received}$ = % total solids content of the original sample (prior to any preparation steps) on a 105^{o}C dry weight basis, as determined by the LAP-001.
T_{prep} = % total solids content of the sample as determined during the preparation of the sample for analysis (by lyophilization, oven-drying, or air drying).
T_{final} = % total solids content of the prepared sample used in this lignin analysis, on a 105^{o}C dry weight basis, as determined by the LAP-001.

Note: If the sample used in the acid-insoluble lignin analysis required no preparation (analyzed as received), then T_{prep}=100% and T_{final}=$T_{as\ received}$. If a sample was prepared by drying at 105^{o}C prior to analysis, then T_{prep}=$T_{as\ received}$ and T_{final}=100%.

3.68 For an extracted sample, it may be necessary to report the results on an extractives-free

105°C dry weight basis or on an as received (whole sample) 105°C dry weight basis, or both.

3.68.1 Calculate % acid-insoluble residue on an extractives-free basis as follows:

$$\%\ acid\text{-}insoluble\ residue_{extractives\text{-}free} = \frac{W_2 - W_3}{W_1 \times \frac{\%T_{final}}{100\%}} \times 100\%$$

Where:
W_1 = initial weight of extracted sample
W_2 = weight of crucible, acid-insoluble residue, acid-insoluble ash
W_3 = weight of crucible and acid-insoluble ash
$\%T_{final}$ = % total solids of the extracted sample determined at 105°C as described by the Standard Method for the Determination of Total Solids in Biomass.

3.68.2 Correct the acid-insoluble residue value calculated above on an extractives-free basis, to an as received (whole sample) 105°C dry weight basis:

$$\%\ acid\text{-}insoluble\ residue_{whole\ sample} = \%\ AIR_{extractives\text{-}free} \times \frac{(100\% - \%\ extractives)}{100\%}$$

Where:
$\%\ AIR_{extractives\text{-}free}$ = % acid-insoluble residue on an extractives-free 105°C dry weight basis, as determined in the previous step
$\%\ extractives$ = % extractives in the sample extracted as described in the Standard Method for the Determination of Extractives in Biomass.

14. Report

3.69 Report the percent acid-insoluble lignin, to two decimal places, on a 105°C dry weight basis, and cite the reporting basis.

3.70 For replicate analyses of the same sample, report the average, standard deviation, and relative percent difference (RPD).

15. Precision and Bias

3.71 Data obtained by replicate testing of a hybrid poplar in one laboratory gave a standard deviation in Klason lignin content of 0.32% and a CV% of 1.26%.

3.72 Data obtained by replicate testing of a hybrid poplar sample in six different laboratories gave a standard deviation of 2.37% and a CV% of 9.92%.

16. Quality Control

3.73 *Reported significant figures:* The acid-insoluble lignin results will be reported with two decimal places, on a 105^{o}C dry weight basis.

3.74 *Replicates:* All samples and all method verification standards are to be analyzed in duplicate, at minimum.

3.75 *Blank:* A blank crucible is to be run through the analysis. The dish is to be weighed empty, ashed and reweighed. The difference in weight must be less than the equivalent of a 0.5% error.

3.76 *Relative percent difference criteria:* The RPD must be less than 3.4%. If the RPD is too large, the sample will be rerun.

3.77 *Method verification standard:* A method verification standard must be run in duplicate with every batch.

3.78 *Calibration verification standard:* Not applicable.

3.79 *Sample size:* Approximately 0.6 grams of sample is required for conducting duplicate analyses by Procedure A. Two grams will be required for Procedure B. If there is insufficient sample, the result will be flagged and the lack of precision will be noted.

3.80 *Sample storage:* Wet samples, prior to preparation, must be stored in the refrigerator. Samples that have been prepped by extraction, lyophilization, or oven drying must be stored in tightly sealed containers or in a desiccator.

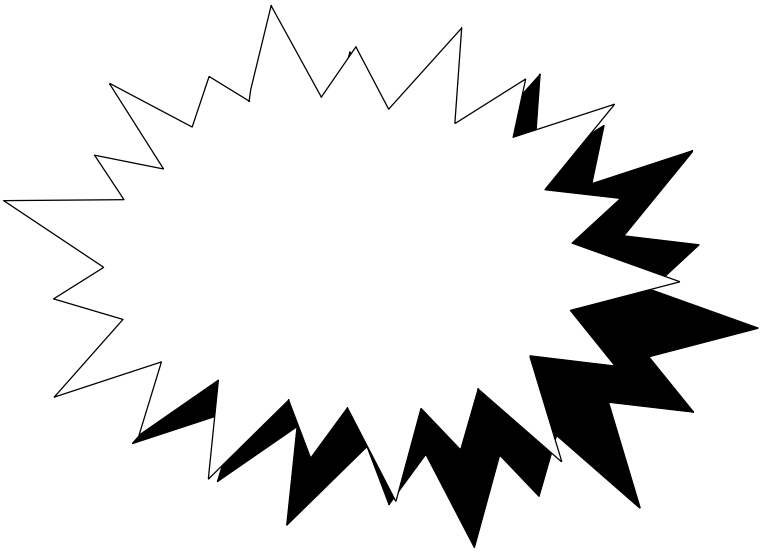
3.81 *Standard storage:* Not applicable.

3.82 *Standard preparation:* Not applicable.

3.83 *Definition of a batch:* Any number of samples which are analyzed together and

recorded together. Samples within a batch must be of the same matrix. The maximum size of a batch would be limited by the equipment constraints. A batch cannot be larger than what is practical with the equipment.

3.84 *Control charts:* The result of each replicate analysis of the method verification standard is recorded along with the average, RPD, and a laboratory book/page reference. The average value obtained for each analysis of the method verification standard is to be control charted.



Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-004

Procedure Title: Determination of Acid-Soluble Lignin in Biomass

Author: Tina Ehrman

Date: **9/9/96**

ISSUE DATE: 9/25/96 **SUPERSEDES:** 8/19/92

Determination of Acid-Soluble Lignin in Biomass

Laboratory Analytical Procedure #004

1. Introduction

1.1 The residue remaining after extensive acid hydrolysis of a biomass sample, corrected for its ash content, is referred to as acid-insoluble lignin. This value, however, does not represent the total lignin content of the sample. A small portion of the lignin is solubilized during the hydrolysis procedure. This lignin fraction is referred to as acid-soluble lignin (ASL) and may be quantified by ultraviolet spectroscopy.

2. Scope

2.1 This procedure describes a spectrophotometric method for determining the amount of lignin solubilized upon hydrolysis of a biomass sample. The protocol utilizes the hydrolyzate generated by LAP-002, "Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography", or by LAP-003, "Determination of Acid-Insoluble Lignin in Biomass".

2.2 Sample material suitable for this procedure include hard and soft woods, herbaceous materials (such as switchgrass and sericea), agricultural residues (such as corn stover, wheat straw, and bagasse), waste-paper (such as office waste, boxboard, and newsprint), washed acid- and alkaline-pretreated biomass, and the solid fraction of fermentation residues. All results are reported relative to the 105±C oven-dried weight of the sample. In the case of extracted materials, the results may also be reported on an extractives-free basis.

2.3 Liquid process samples may also be analyzed by this technique to give an estimate of their acid-soluble lignin content. The values generated must be viewed as estimates only since many other components present in liquid process samples will also absorb at the analysis wavelength and will bias the results high.

2.4 All analyses shall be preformed according to the guidelines established in the Ethanol Project Quality Assurance Plan (QAP).

3. References

3.1 Ethanol Project Laboratory Analytical Procedure #002, "Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography".

3.2 Ethanol Project Laboratory Analytical Procedure #003, "Determination of Acid-Insoluble Lignin in Biomass".

3.3 Ethanol Project Laboratory Analytical Procedure #010, "Determination of Extractives in Biomass".

3.4 Kaar, W.E., and D.L. Brink. 1991. "Simplified Analysis of Acid Soluble Lignin." *Journal of Wood Chemistry and Technology*, 11(4):465-477.

3.5 TAPPI Test Method T250, "Acid-Soluble Lignin in Wood and Pulp." *In Tappi Test Methods.* Atlanta, GA: Technical Association of the Pulp and Paper Industry.

4. Significance and Use

4.1 The acid-soluble lignin determination is used in conjunction with other assays to determine the total composition of biomass samples.

5. Interferences

5.1 Any component besides acid-soluble lignin which is present in the hydrolyzate and which absorbs at the analytical wavelength of 205 nm will cause the results to be biased high. This problem will be most severe with liquid process samples which have been shown to contain components which absorb at 205 nm.

6. Apparatus

6.1 Spectrophotometer, suitable for measuring absorbance at 205 nm.

7. Reagents and Materials

7.1 Sulfuric acid, 4% w/w, prepared by diluting 3.00 ± 0.01 mL of 72% w/w H_2SO_4 with 84.00 ± 0.04 mL of deionized water.

> Note: For hydrolyzates generated from solid biomass samples taken through LAP-002 or LAP-003, the sulfuric acid used to prepare the 4% acid solution must be from the same prepared batch of 72% w/w H_2SO_4 used to prepare the sample.

7.2 Water, 18 megohm deionized.

7.3 Matched pair of quartz cuvettes with a 1-cm path length.

7.4 Glass test tubes, of a size suitable for making dilutions.

7.5 Adjustable pipettors of various sizes with the appropriate disposable tips.

8. ES&H Considerations and Hazards

8.1 72% H_2SO_4 is very corrosive and must be handled carefully.

8.2 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

9. Procedure

9.1 Set up and calibrate the spectrophotometer following the protocols recommended in the instrument manual.

9.2 Measure the absorbance of the hydrolyzate at 205 nm, using the 1-cm light path cuvette. A 4% solution of H_2SO_4 should be used as a reference blank.

9.3 If the absorbance reading exceeds 0.7, the sample must be diluted. Dilute the sample so the resulting absorbance reading falls between 0.2 and 0.7. The 4% H_2SO_4 must be diluted in the same ratio as the sample and used as the reference blank for this repeat analysis.

9.4 Repeat the analysis on a second aliquot of the hydrolyzate. Each sample must be analyzed in duplicate, including the hydrolyzates generated from the analysis of the LAP-002 or LAP-003 method verification standard.

10. Calculations

10.1 An absorptivity (extinction coefficient) value of 110 L/g-cm is used to calculate the amount of acid-soluble lignin present in the hydrolyzate. The 205 nm absorptivities reported for most woods fall in the range of 88 to 113 L/g-cm. The value of 110 L/g-cm used in this protocol is consistent with the value used in the TAPPI procedure and represents an average of values found for different woods and pulps.

> Note: An absorptivity for a given type of biomass may be determined using the "reKlasonation" protocol described in Kaar and Brink (1991).

10.2 For a liquid process sample, an estimate can be made of the amount of acid-soluble lignin present as follows:

$$ASL, estimated\ (g/L) = \frac{A}{b\ x\ a}\ x\ df$$

Where:

A= absorbance at 205 nm.
df = dilution factor.
b = cell path length, 1 cm.
a = absorptivity, equal to 110 L/g-cm unless experimentally determined for a given biomass material.

10.3 For a solid biomass sample, the percent acid soluble lignin on a 105°C dry weight or on an extractives free basis is calculated as follows:

$$\%\ ASL = \frac{\frac{A}{b\ x\ a}\ x\ df\ x\ V\ x\ \frac{L}{1000\ mL}}{\frac{W\ x\ T_{final}}{100}}\ x\ 100$$

Where:

A = absorbance at 205 nm.
df = dilution factor.
b = cell path length, 1 cm.
a = absorptivity, equal to 110 L/g-cm unless experimentally determined for a given biomass material.
V = filtrate volume, this volume will either be 87 mL, if the sample is the hydrolyzate from the carbohydrate analysis (LAP-002) or the LAP-003 summative acid-insoluble lignin protocol, or will be equal to the weight of the filtrate obtained in the LAP-003 Klason lignin protocol expressed in mL.
W = initial biomass sample weight in grams (from LAP-002 or LAP-003).
$\%T_{final}$ = % total solids content of the biomass sample (as received or after extraction), on a 105°C dry weight basis, as determined during the LAP-002 or LAP-003 analysis.

10.4 For an extracted biomass sample, the percent acid-soluble lignin value, calculated above, can be converted to an as received (whole sample) 105°C dry weight basis as follows:

$$\%\ ASL_{whole\ sample} = \%\ ASL_{extractives\text{-}free}\ x\ \frac{(100\% - \%\ extractives)}{100\%}$$

Where: % $ASL_{extractives\text{-}free}$ = % acid-soluble lignin on an extractives-free 105°C dry weight basis, as determined in the previous step.
% extractives = % extractives in the extracted sample as described in LAP-010, "Standard Method for the Determination of Extractives in Biomass".

11. Report

11.1 For solid biomass samples, report the percent acid-soluble lignin present in the sample, to two decimal places, on a whole sample 105°C dry weight basis or on an extractives-free basis. For liquid process samples, report the acid-soluble lignin content as an estimate, to two decimal places. Cite the basis used in the report.

11.2 For replicate analyses of the same sample, report the average, standard deviation, and relative percent difference (RPD).

12. Precision

12.1 Data obtained by replicate testing of a hybrid poplar in one laboratory gave a standard deviation of 0.12% and a CV of 5.41%.

12.2 Data obtained by replicate testing of a hybrid poplar sample in six different laboratories gave a standard deviation of 0.98% and a CV of 41%.

13. Quality Control

13.1 *Reported significant figures:* For solid biomass samples, report the percent acid-soluble lignin present in the sample, to two decimal places, on a whole sample 105°C dry weight basis or on an extractives-free basis. For liquid process samples, report the acid-soluble lignin content as an estimate, to two decimal places. Cite the basis used in the report.

13.2 *Replicates:* At minimum, all samples and the method verification standard are to be analyzed in duplicate.

13.3 *Blank:* Dilute the 4% sulfuric acid solution in the same manner as the sample and use as the reference blank in the spectrophotometer.

13.4 *Relative percent difference criteria:* The RPD must be less than 15.5%. If the RPD is too large, the sample must rerun.

13.5 *Method verification standard:* A method verification standard must be run in duplicate with every batch. This method utilizes the hydrolyzate generated by LAP-002 or LAP-003 from a well characterized standard material suitable for analysis. For example, NIST 8492 (*Populus deltoides*) is used as the MVS in compositional analysis of hardwoods.

13.6 *Calibration verification standard:* Not applicable.

13.7 *Sample size:* A minimum of 5 mL of hydrolyzate generated by LAP-002 or LAP-003 are required for duplicate analyses. If there is insufficient sample, the result will be flagged and the lack of precision will be noted.

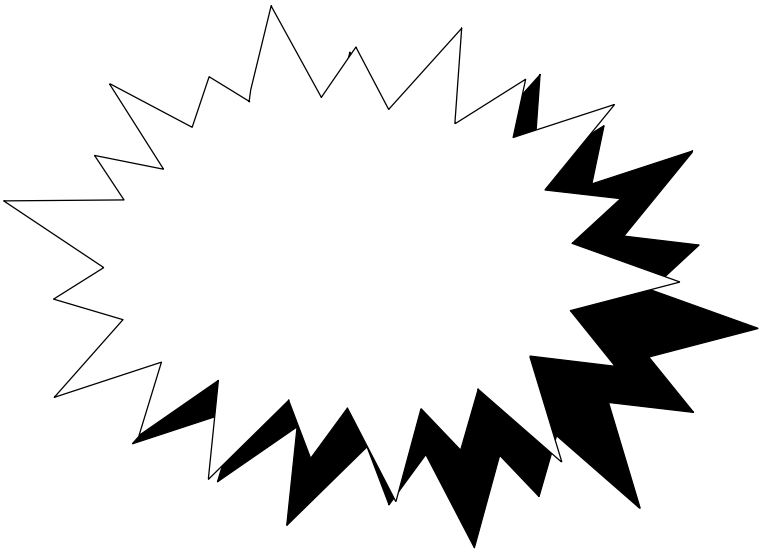
13.8 *Sample storage:* Samples shall be stored in an airtight container and refrigerated. They must be analyzed within 24 hours, and preferably within 6 hours, of the hydrolysis step of LAP-002 or LAP-003.

13.9 *Standard storage:* Not applicable.

13.10 *Standard preparation:* Not applicable.

13.11 *Definition of a batch:* Any number of samples which are analyzed and recorded together. The maximum size of a batch would be limited by the equipment constraints. A batch cannot be larger than what is practical with the equipment.

13.12 *Control charts:* The result of each replicate analysis of the method verification standard is recorded along with the average, RPD, and a laboratory book/page reference. The average value obtained for each analysis of the method verification standards is to be control charted.



Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-005

Procedure Title: Standard Method for Ash in Biomass

Author: Tina Ehrman

Date: 4/28/94

ISSUE DATE: 4-28-94 **SUPERSEDES:** 8/18/92

Standard Method for Ash in Biomass

Laboratory Analytical Procedure #005

1 Introduction

1.1 This procedure has been adopted by ASTM as an ASTM Standard Test Method for the determination of ash in biomass.

2. Scope

2.1 This test method covers the determination of ash, expressed as the percentage of residue remaining after dry oxidation (oxidation at 550 to 600^{o}C), of hard and soft woods, herbaceous materials (such as switchgrass and sericea), agricultural residues (such as corn stover, wheat straw, and bagasse), wastepaper (such as office waste, boxboard, and newsprint), acid and alkaline pretreated biomass, and the solid fraction of fermentation residues. All results are reported relative to the 105^{o}C oven-dried weight of the sample.

2.2 All analysis shall be performed according to the Ethanol Project Quality Assurance Plan (QAP).

3. References

3.1 TAPPI Test Method T211, "Ash in Wood and Pulp." *In Tappi Test Methods.* Atlanta, GA: Technical Association of the Pulp and Paper Industry.

3.2 Moore, W., and D. Johnson. 1967. *Procedures for the Chemical Analysis of Wood and Wood Products*. Madison, WI: U.S. Forest Products Laboratory, U.S. Department of Agriculture.

4. Terminology

4.1 Ash - The inorganic residue left after ignition at 575^{o}C.

5. Significance and Use

5.1 The ash content is an approximate measure of the mineral content and other inorganic matter in biomass.

5.2 The ash content is used in conjunction with other assays to determine the total composition of biomass samples.

6. Apparatus

6.1 *Aluminum weighing pans or crucibles, 50 mL* - If crucibles are used, platinum crucibles are preferred, but silica or porcelain crucibles may be used.

6.2 *Muffle furnace* - An electric furnace is recommended for igniting the wood sample. A furnace fitted with an indicating pyrometer, so that the desired temperature can be maintained, is preferable.

6.3 *Analytical balance,* sensitive to 0.1 mg.

6.4 *Desiccator*.

6.5 *Drying oven,* with temperature control of $105 \pm 2^{o}C$.

7 ES&H Considerations

7.1 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

8. Test Specimen

8.1 Test specimens suitable for analysis by this procedure are as follows:
- biomass feedstocks,
- pretreated biomass,
- the solids fraction of fermentation residues.

8.2 Samples must be dried at $105^{o}C$ according to the Laboratory Analytical Procedure #001, Determination of Total Solids and Moisture in Biomass, prior to ash analysis. Air-dried material can be used in place of $105^{o}C$ dried material, but the weight of the material must be corrected for its moisture content prior to calculating the ash.

8.3 The test specimen shall consist of approximately 0.5 to 1.0 g of sample obtained in such a manner to ensure that it is representative of the entire lot of material being tested. For $105^{o}C$ dried samples containing large particles or chunks, it is recommended that the sample be ground or milled to reduce the size of the large pieces to less then 1 mm in diameter. The sample is then redried at $105^{o}C$ prior to testing.

9. Procedure

9.1 Mark a pan or crucible with a unique identification using a porcelain marker, place it in the muffle furnace, and bring to constant weight by igniting at $575 \pm 25^{o}C$. Remove the pan or crucible from the furnace, cool to room temperature in a desiccator, and weigh to the nearest 0.1 mg. Record this weight as the tare weight. Keep the pan or crucible in a desiccator until used.

> Note: For an aluminum pan, two hours of heating at $575 \pm 25^{o}C$ will be sufficient to bring the pan to constant weight. With a crucible, however, the following procedure is used: Place the crucible in the furnace at $575 \pm 25^{o}C$ for three hours. Remove the crucible and place in a desiccator. Allow the crucible to cool to room temperature and then weigh the crucible to the nearest 0.1 mg. Record this weight. After weighing, return the crucible to the furnace for one hour at $575 \pm 25^{o}C$, cool again in the desiccator, and reweigh. Repeat this step until the weight of the crucible varies by less than 0.3 mg from the previous weighing. Record this final weight as the crucible tare weight.

9.2 Weigh approximately 0.5 to 1.0 g, to the nearest 0.1 mg, of a test specimen into the tared pan or crucible. If the sample being analyzed is a $105^{o}C$ dried test specimen, the sample should be stored in a desiccator until use. Record the weight (container plus sample minus tare weight of container) as the initial weight of the test specimen, W_2.

> Note: For air dried samples, we recommend that samples for moisture determination should be weighed out at the same time as the samples for the ash determination. If this is done at a later time it can introduce an error in the calculation because ground biomass can rapidly gain or lose moisture when exposed to the atmosphere.

9.3 Place the container and contents in the muffle furnace and ignite at $575 \pm 25^{o}C$ for a minimum of three hours, or until all the carbon is eliminated. Heat slowly at the start to avoid flaming. If the sample tends to flare up, the container should be partially covered during this step. Avoid heating above the maximum stated temperature. Protect the test container from strong drafts at all times to avoid mechanical loss of test specimen.

> Note: For test specimens containing high amounts of ash (greater than 5% by weight), it will be necessary to increase the time in the furnace to overnight to ensure complete elimination of the carbon. This ignition time period should not exceed 24 hours.

9.4 Remove the pan or crucible with its contents to a desiccator, cool to room temperature, weigh to the nearest 0.1 mg, and record this weight. Repeat the heating for one hour periods until the weight after cooling is constant to within0.3 mg. Record the final weight of the ash, W_1, as the container plus ash weight minus container tare weight.

10. Calculations

10.1 For 105°C dried materials, calculate the percentage of ash, based on the initial weight of the test specimen, as follows.

$$\text{Ash, \%} = (W_1/W_2) \times 100$$

where: W_1 = weight of ash, and
W_2 = initial weight of 105°C dried sample.

10.2 For air dried samples, the following calculation may be used to report the results on a 105°C dried weight basis:

$$\text{Ash, \%} = (W_1 / (W_2 \times T/100)) \times 100$$

where:

W_1 = weight of ash,
W_2 = initial weight of sample, and
T = percent total solids of sample, on a 105°C oven-dried weight basis, as determined by LAP #001.

11. Report

11.1 Report the result to two decimal places, as a percentage of the sample's 105°C dried weight, and cite the basis used in the calculation.

12. Precision and Bias

12.1 Data obtained by replicate testing of a hybrid poplar in one laboratory gave a standard deviation in ash content of 0.05% and a CV% of 3.88%. Replicate testing of a National Institute of Standards and Technology (NIST) #8494 wheat straw gave a standard deviation of 0.20% and a CV% of 1.95%.

12.2 Data obtained by replicate testing of a hybrid poplar sample in six different laboratories gave a standard deviation in ash content of 0.11% and a CV% of 9.24%

13. Quality Control

13.1 *Reported significant figures:* The ash results will be reported as a percentage with two decimal places.

13.2 *Replicates:* All samples and all method verification standards are to be analyzed in duplicate.

13.3 *Blank:* An empty aluminum dish or crucible is to be run through the analysis. The dish is to be weighed empty, ashed, and reweighed. The difference in weight must be less than the equivalent of a 0.5% error.

13.4 *Relative percent difference criteria:* The %RPD must be less than 15.5% if the average ash content is less than 2%. The %RPD must be less than 5.0% if the average ash content is greater than 2%. If the %RPD is too large, the sample will be rerun.

13.5 *Method verification standard:* A method verification standard must be run in duplicate with every batch. The results of method verification analyses must be control charted.

13.6 *Calibration verification standard:* Not applicable.

13.7 *Sample size:* Approximately two grams are required for duplicate analyses. If there is insufficient sample, the result will be flagged and the lack of precision will be noted.

13.8 *Sample storage:* The oven dried sample will be stored in a desiccator.

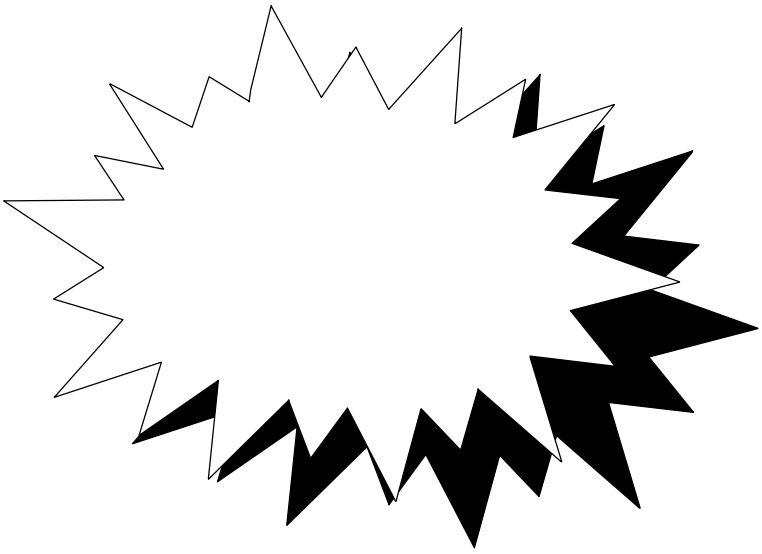
13.9 *Standard storage:* Not applicable.

13.10 *Standard preparation:* Not applicable.

13.11 *Definition of a batch*: Any number of samples which are analyzed together and recorded together. Samples within a batch must be of the same matrix. The maximum size of a batch would be limited by the equipment constraints. A batch cannot be larger than what is practical with the equipment.

13.12 *Control charts:* The results of analysis of method verification standards are recorded. Each result from the duplicate analysis is recorded along with the average, %RPD, and a laboratory book/page reference.

13.13 *Other:* All aluminum pans or crucibles shall be preashed. Because the samples and containers quickly pick up moisture from the air, all the weights shall be taken immediately after the items are removed from the desiccator.



Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-006

Procedure Title: Measurement of Cellulase Activities

Author: Bill Adney and John Baker

Date: **7-18-96**

ISSUE DATE: 8/12/96 **SUPERSEDES:** 8/19/92

Measurement of Cellulase Activities

Laboratory Analytical Procedure #006

1. Introduction

1.1 The following method describes a procedure for measurement of cellulase activity using International Union of Pure and Applied Chemistry (IUPAC) guidelines (1). The procedure has been designed to measure cellulase activity in terms of "filter-paper units" (FPU) per milliliter of original (undiluted) enzyme solution. For quantitative results the enzyme preparations must be compared on the basis of significant and equal conversion. The value of 2.0 mg of reducing sugar as glucose from 50 mg of filter paper (4% conversion) in 60 minutes has been designated as the intercept for calculating filter paper cellulase units (FPU) by IUPAC.

1.2 It is extremely important to keep in mind that the FPU is defined only at this extent of conversion. Reducing sugar yield is not a linear function of the quantity of enzyme in the assay mixture; as discussed by Ghose (1987), twice the amount of enzyme would not be expected to yield twice the reducing sugar in equal time. The assay procedure therefore involves finding a dilution of the original enzyme stock such that a 0.5 mL aliquot of the dilution will catalyze 4% conversion in 60 minutes (or, in practical terms, finding two dilutions that bracket the 4%-conversion point so closely that the required dilution can be obtained, with reasonable accuracy, by interpolation) and then calculating the activity (in FPU/mL) of the original stock from the dilution required. Further comments on the required calculations, and their significance, are to be found in the Appendix.

1.3 Assay mixtures may in some cases contain reducing sugars unrelated to hydrolysis of substrate glycosidic bonds by the enzyme. Culture filtrates to be assayed for cellulase may contain nutrient sugars, and the reducing ends of the cellulose polymers of the substrate may sometimes be measurable as glucose equivalents before any enzyme attack. For this reason, controls consisting of (a) enzyme without substrate and b) substrate without enzyme are included with all enzyme assays and sample values are corrected for any blank values.

2. Scope

2.1 This procedure is only appropriate for the determination of FPU activity in a cellulase preparation as defined by the IUPAC procedure as outlined above.

3. References

3.1 Ghose, T.K. 1987. "Measurement of Cellulase Activities." *Pure & Appl. Chem.* 59: 257-268.

3.2 Miller, G.L. 1959. "Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar." *Anal. Chem*. 31:426-428.

4. Significance and Use

4.1 This procedure follows IUPAC guidelines and determines enzyme activity as filter paper units in a cellulase preparation.

5. Apparatus

5.1 Water bath capable of maintaining $50^{o}C \pm 0.1^{o}C$.

5.2 Spectrophotometer suitable for measuring absorbance at 540 nm.

6. Reagents and Materials

6.1 DNS Reagent

Mix:	Distilled water	1416 mL
	3,5 Dinitrosalicylic acid	10.6 g
	Sodium hydroxide	19.8 g

Dissolve above, then add:

Rochelle salts (sodium potassium tartrate)	306 g
Phenol (melt at $50^{o}C$)	7.6 mL
Sodium metabisulfite	8.3 g

Titrate 3 mL sample with 0.1 N HCl to the phenolphthalein endpoint. It should take 5-6 mL of HCl. Add NaOH if required (2 g = 1 mL 0.1 N HCL).

6.2 **Citrate Buffer**: For *Trichoderma reesei*, cellulase assays are carried out in 0.05 M citrate buffer pH 4.8. For other cellulase enzymes, the pH and the assay temperature may be different. The assay conditions must be defined when reporting results.

Citric acid monohydrate	210 g
DI water	750 mL
NaOH - add until pH equals 4.3	50 to 60 g

Dilute to 1 L and check pH. If necessary add NaOH until the pH is 4.5. When the 1 M stock citrate buffer stock is diluted with water to 50 mM the pH should be 4.8. After diluting the citrate buffer check and adjust the pH if necessary to pH 4.8.

7. ES&H Considerations and Hazards

7.1 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

7.2 Care must be taken when working with phenol, which is toxic and corrosive.

8. Procedure for the Filter Paper Assay for Saccharifying Cellulase

8.1 The detection of glycosidic bond cleavage by this method involves the parallel and identical treatment of three categories of experimental tubes (assay mixtures, blanks and controls, and glucose standards), prepared as detailed below. The substrate is a 50 mg Whatman No. 1 filter paper strip (1.0 x 6.0 cm).

8.2 Enzyme assay tubes:

8.2.1 Place a rolled filter paper strip into each 13 x 100 test tube.

8.2.2 Add 1.0 mL 0.05 M Na-citrate, pH 4.8 to the tube; the buffer should saturate the filter paper strip.

8.2.3 Equilibrate tubes with buffer and substrate to 50^{o}C.

8.2.4 Add 0.5 mL enzyme diluted appropriately in citrate buffer. At least two dilutions must be made of each enzyme sample, with one dilution releasing slightly more than 2.0 mg of glucose (absolute amount) and one slightly less than 2.0 mg of glucose. Target 2.1 and 1.9 mg glucose, respectively, for these two dilutions. Depending on the enzyme these targets may be hard to achieve and additional dilutions must be run.

8.2.5 Incubate at 50^{o}C for exactly 60 min.

8.2.6 At the end of the incubation period, remove each assay tube from the 50^{o}C bath and stop the enzyme reaction by immediately adding 3.0 mL DNS reagent and mixing.

8.3 Blank and controls:

8.3.1 Reagent blank: 1.5 mL citrate buffer.

8.3.2 Enzyme control: 1.0 mL citrate buffer + 0.5 mL enzyme dilution (prepare a separate control for each dilution tested).

8.3.3 Substrate control: 1.5 mL citrate buffer + filter-paper strip.

8.4 Glucose standards:

8.4.1 A working stock solution of anhydrous glucose (10 mg/mL) should be made up. Aliquots of this working stock should be tightly sealed and stored frozen. The standard should be vortexed after thawing to ensure adequate mixing.

8.4.2 Dilutions are made from the working stock in the following manner:

1.0 mL + 0.5 mL buffer = 1:1.5 = 6.7 mg/mL (3.35 mg/0.5 mL).
1.0 mL + 1.0 mL buffer = 1:2 = 5 mg/mL (2.5 mg/0.5 mL).
1.0 mL + 2.0 mL buffer = 1:3 = 3.3 mg/mL (1.65 mg/0.5 mL).
1.0 mL + 4.0 mL buffer = 1:5 = 2 mg/mL (1.0 mg/0.5 mL).

8.4.3 Glucose standard tubes should be prepared by adding 0.5 mL of each of the above glucose dilutions to 1.0 mL of citrate buffer in a 13 x 100 mm test tube.

8.4.4 Blanks, controls and glucose standards should be incubated at 50^{o}C along with the enzyme assay tubes, and then "stopped" at the end of 60 minutes by addition of 3.0 mL of DNS reagent.

8.5 Color development (Miller, 1959):

8.5.1 Boil all tubes for exactly 5.0 minutes in a vigorously boiling water bath containing sufficient water to cover the portions of the tubes occupied by the reaction mixture plus reagent. All samples, controls, blanks, and glucose standards should be boiled together. After boiling, transfer to a cold ice-water bath.

8.5.2 Let the tubes sit until all the pulp has settled, or centrifuge briefly. Dilute all tubes (assays, blanks, standards and controls) in water (0.200 mL of color-developed reaction mixture plus 2.5 mL of water in a spectrophotometer cuvette works well, use the pipettor to mix by drawing the mixture into the pipettor tip repeatedly). Determine color formation by measuring absorbance against the reagent blank at 540 nm. With this dilution the glucose standards described above should give absorbance in the range of 0.1 to 1.0 A.

9. Calculations

9.1 Construct a linear glucose standard curve using the absolute amounts of glucose (mg/0.5 mL) plotted against A_{540}. The data for the standard curve should closely fit a calculated straight line, with the correlation coefficient for this straight line fit being very near to one. Verify the standard curve by running a calibration verification standard, an independently prepared solution of containing a known amount of glucose which falls about midpoint on the standard curve.

9.2 Using this standard curve determine the amount of glucose released for each sample tube after subtraction of enzyme blank.

9.3 Estimate the concentration of enzyme which would have released exactly 2.0 mg of glucose by means of a plot of glucose liberated against the logarithm of enzyme concentration (refer to the example in Appendix B, which uses semilogarithmic graph paper). To find the required enzyme concentration take two data points that are very close to 2.0 mg and draw a straight line between them, use this line to interpolate between the two points to find the enzyme dilution that would produce exactly 2.0 mg glucose equivalents of reducing sugar. Appendix B presents an example.

Note: In this plot, and in the equation below for calculating FPU, the term "enzyme concentration" refers to the proportion of the original enzyme solution present in each enzyme dilution (i.e., the number of mL of the original solution present in each mL of the dilution).

9.4 Calculate FPU:

$$\textit{Filter Paper Activity} = \frac{0.37}{\textit{[enzyme] releasing 2.0 mg glucose}}\ \textit{units/ml}$$

Where [enzyme] represents the proportion of original enzyme solution present in the directly tested enzyme dilution (that dilution of which 0.5 mL is added to the assay mixture). For the derivation of the FPU see Ghose (1987) and Appendix A.

9.5 Refer to Appendix B for an example for calculating IUPAC-FPU.

10. Precision and Bias

10.1 Precision can be measured only by the closeness of repeated measurements of the same quantity of enzyme. This procedure, if carefully followed, should give the same approximate numerical readings as obtained by other laboratories using the same procedure. Precision in filter paper assays may be affected by the inherent physical properties of cellulase preparations.

11. Quality Control

11.1 *Reported significant figures:* Typically results are reported as whole integers along with the standard deviation. The assay conditions must be defined when reporting the results.

11.2 *Replicates:* Run each dilution in triplicate.

11.3 *Blank:* As described in the section "Blank and controls".

11.4 *Relative percent difference criteria:* Not defined; dependent on the enzyme being tested.

11.5 *Method verification standard:* Not available since enzymes change over time.

11.6 *Calibration verification standard:* A calibration verification standard shall be independently prepared and analyzed as described in the section "Calculations".

11.7 *Sample size:* Dependant upon enzyme concentration.

11.8 *Sample storage:* Dependant upon source of enzyme. Manufacturer's instructions should be followed.

11.9 *Standard storage:* Store frozen at -20^{o}C or prepare fresh batch; shake vigorously prior to use.

11.10 *Standard preparation:* As described in the section "Glucose standards".

11.11 *Definition of a batch*: Run all standards, blanks, and samples together in one batch. The size of the batch may be limited by instrument constraints and should not be larger than what is practical to handle together.

11.12 *Control charts:* Not applicable.

11.13 *Others:* Not applicable.

12. **Appendix A:** Numerical Values in Equation Used to Calculate Filter Paper Activity

The practical bottom line is that if the assays are set up according to the instructions, and the calculations are carried out using the equation presented in the calculations section, the results obtained will correspond to the generally accepted activities in "filter paper units" that would be obtained by other laboratories around the world, were these other laboratories to test the same enzyme solution. For those workers interested in the derivation of this equation, and of the "filter paper unit", the following comments may be helpful in conjunction with Ghose (1987).

The numerator (0.37) in the equation is derived from the factor for converting the 2.0 mg of "glucose-equivalents" generated in the assay to mmoles of glucose (2.0 ÷ 0.18016), from the volume of the enzyme being tested that is used in the assay (0.5 mL), and from the incubation time (60 minutes) required for generation of the reducing equivalents.

$$\frac{(2.0\ mg\ glucose\ /\ 0.18016\ mg\ glucose/mmol)}{(0.5\ mL\ enzyme\ dilution\ x\ 60\ minutes)} = 0.37\ mmol/minute - mL$$

Thus,
Because the "enzyme concentration" in the denominator of the equation is a dimensionless number (equal to the ratio of the enzyme concentration in the 0.5 mL of enzyme dilution added to the assay to the enzyme concentration in the original solution, for which FPU values are desired), the right side of equation therefore winds up with units (mmol $min^{-1}mL^{-1}$) that look like "International Units per mL" (I.U./mL). Ghose himself points out, however, that "because the FPU assay is non-linear, the use of the International Unit *per se* is incorrect as this unit is based on initial velocities, i.e., linear reactions in which the product is produced at the same rate during each and every minute of the reaction." The author goes on to recommend that FPU values for a given cellulase solution be given simply as "units/mL".

"Definition" of the "Filter Paper Unit":

As a result of the above choice of numerical values, the "Filter Paper Unit" is not actually explicitly defined. What is defined is the quantity 0.1875 FPU, which is that quantity of enzyme activity that, when assayed according to the instructions contained herein, will produce reducing sugar equivalent to 2.0 mg of glucose. One can verify this from the equation presented in the calculations section by assuming that the enzyme solution being tested needs no dilution to yield reducing sugar equivalent to 2.0 mg of glucose (i.e., the "enzyme concentration" ratio in the denominator is equal to 1.0), in which case the activity of the solution being tested is measured as 0.37 filter paper units per mL. Inasmuch as 0.5 mL of this solution was used in the assay, the absolute quantity of enzyme activity that is present in the assay (and to which the observed effect can be ascribed) is 0.1875 FPU.

To put it another way, we have a defined method for measuring the activity of a cellulase solution containing 0.1875 filter paper units per 0.5 mL assay aliquot (0.37 filter paper units per mL of enzyme solution) but we do not have method for measuring the filter paper activity of solutions with any other value. Solutions containing more than 0.37 "units" per mL must therefore be diluted to this standard value to be measured, and solutions containing less than 0.37 "units" per mL (reducing sugar produced in 60 minutes is less than that equivalent to 2.0 mg of glucose) cannot be assigned "filter paper unit" activities at all. These latter "sub-2.0-mg" solutions either must be concentrated before assay, or the activities should not be reported as "filter paper units" at all, but should be reported instead as "mmoles glucose equivalents released per minute averaged over 60 minutes."

Ghose (1987) explains the special circumstances involved in measurement of "filter paper activity", and workers are urged to pay close attention to the text of the paper (especially the text surrounding the equations on page 263 of the reference) rather than just "lifting" the equations themselves.

13. Appendix B: Example for calculating IUPAC-FPU

13.1 Determination of cellulase activity in a *T. reesei* enzyme preparation using the methods outlined by IUPAC. All enzyme dilutions were made in citrate buffer, pH 4.8, as indicated in the following table from a working enzyme stock solution that had been diluted 1:20 in citrate buffer.

Dilution #	Citrate buffer (ml)	1:20 Enzyme (ml)	Concentration*
1	1650	350	0.00875
2	1700	300	0.00750
3	1800	200	0.00500
4	1850	150	0.00375
5	1900	100	0.00250

*The term "concentration" is used to represent the proportion of the original enzyme solution present in the dilution added to the assay mixture. For example a 1:10 dilution of the 1:20 working stock of enzyme will have a "concentration" of 0.005.

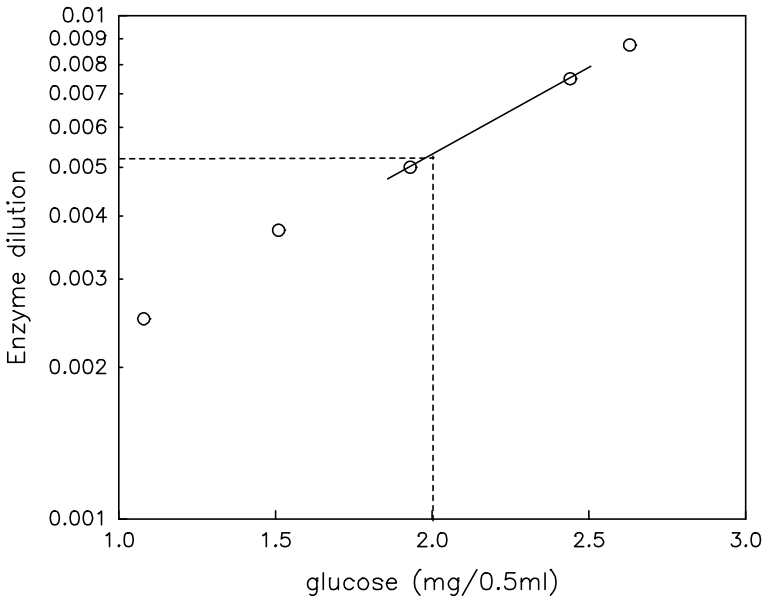
13.2 Dilution of glucose standards and construction of standard curve.

Glucose stock (mL)	Citrate buffer (mL)	Dilution	Concentration	Abs. 540 nm
1.0	0.5	1:1.5	3.35 mg/0.5 mL	0.765
1.0	1.0	1:2	2.50 mg/0.5 mL	0.579
1.0	2.0	1:3	1.65 mg/0.5 mL	0.384
1.0	4.0	1:5	1.00 mg/0.5 mL	0.220

13.3 Glucose concentration of samples as determined from standard curve.

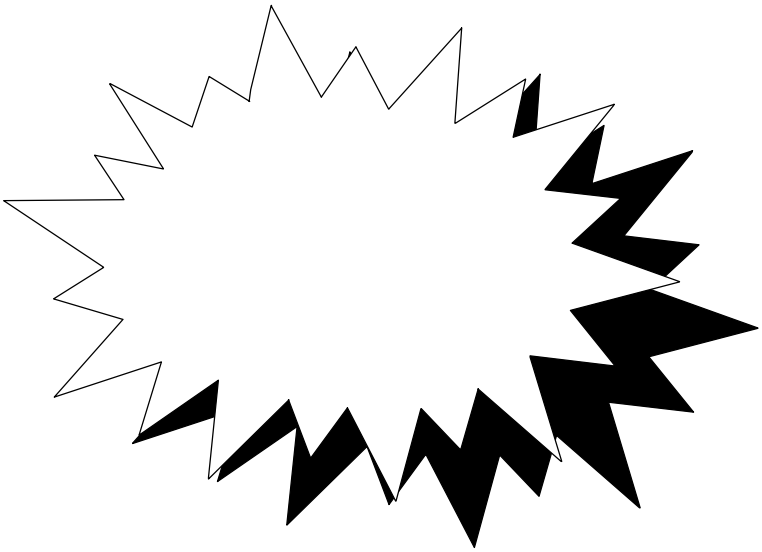
Dilution #	Abs 540 nm	Glucose (ml/0.5 mL)
1	0.603	2.63
2	0.567	2.44
3	0.442	1.93
4	0.346	1.51
5	0.248	1.08

13.4 Determination of the concentration of enzyme which would have released exactly 2.0 mg of glucose by plotting glucose liberated against enzyme concentration.



13.5 Calculation of FPU from graph of dilution vs. glucose concentration.

$$\frac{0.37}{0.0053} = 70\ FPU/mL$$



Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-007

Procedure Title: ***Preparation of Dilute-Acid Pretreated Biomass***

Author: Daniel Hsu

Date: 5/17/95

ISSUE DATE: 5/31/95 **SUPERSEDES:** 3/17/93

Preparation of Dilute-Acid Pretreated Biomass

Laboratory Analytical Procedure #007

1. Introduction

1.1 Lignocellulosic biomass feedstocks, typically, contain large quantities (55-75% by weight) of carbohydrates that are polymers of 5- and 6-carbon sugars. Most or all of these carbohydrates can be converted to ethanol via biotechnology. For bioconversion to occur, however, the polymers must first be broken down into low-molecular-weight, or, essentially, monomeric, sugars, and it is generally accepted that enzymatic hydrolysis is the preferred pathway. Nevertheless, the native (indigenous) cellulose fraction of the carbohydrates is recalcitrant to enzymatic breakdown; therefore, a pretreatment step is required to render it amenable to enzyme attack. Although various pretreatment techniques are under investigation, a batch, dilute sulfuric acid prehydrolysis method is currently employed at NREL to prepare pretreated biomass for fermentation research.

2. Scope

2.1 This procedure describes the batch dilute-acid pretreatment method employed at NREL to prepare pretreated biomass for fermentation research in the Ethanol Project. Biomass is pretreated in a pressure reactor at 160°C for approximately 10 minutes using dilute sulfuric acid. The procedure has been developed as a result of several years of pretreatment research at NREL and has been found to result in prehydrolyzate (the liquid phase resulted from a dilute-acid pretreatment run) of satisfactorily high xylose yield and high pretreated solids enzyme digestibility. Depending on research goals, the pretreated biomass may or may not need to be washed. The operating conditions used in the procedure, however, have not been optimized to maximize the xylose yield, enzyme digestibility, or the ethanol yield that can be obtained through fermentations. Furthermore, the optimal pretreatment conditions are expected to be substrate specific.

2.2 All analyses shall be performed according to the guidelines established in the Ethanol Project Quality Assurance Plan (QAP).

3. References

3.1 NREL Ethanol Project CAT Task Laboratory Analytical Procedure #001, "Standard Method for Determination of Total Solids in Biomass".

3.2 Grohmann, K., Torget, R., and Himmel, M. 1985. "Optimization of Dilute Acid Pretreatment of Biomass." Biotech. Bioeng. Symp. No. 15. 59-80.

3.3 Grohmann, K., Himmel, M., Rivard, C., Tucker, M., Baker, J., Torget, R., and Graboski, M. "Chemical-Mechanical Methods for the Enhanced Utilization of Straw." 1984. Biotech. Bioeng. Symp. No. 14. 137-157.

4. Significance and Use

4.1 Pretreatment is necessary before the carbohydrates in lignocellulosic biomass can be converted to ethanol via biotechnology. Dilute-acid pretreatment is one technique that has been extensively investigated and found to be effective.

5. Apparatus

5.1 Parr Instrument Company pressure reactor system, including either a 2-gal or a 1-L reactor vessel made of Carpenter 20 Cb-3 material, with a matching mixer, jacket electrical heater, and temperature/mixing control unit that comprises a thermocouple, all supported on a movable metal stand. Attached to the head plate of the reactor vessel are a mixer shaft, a pressure gauge, a vent, a valve for chemical injection, a pressure rupture disc, and a thermowell.

5.2 Hoist (required only for the 2-gal system).

5.3 High pressure pump (HPLC pump, Beckman model 110, or equivalent).

5.4 Balance(s).

5.5 pH meter. (**Note: A pH meter must be used if pretreatment of a new type of biomass substrate is performed. If pretreatment of the substrate has been performed previously, pH paper, as specified in Paragraph 6.7, can be used in place of the pH meter.**)

5.6 Stopwatch.

5.7 Tachometer.

6. Reagents and Materials

6.1 Sulfuric Acid, 72% (w/w) (12.00 ± 0.02 M or specific gravity 1.6389 at 15.6°C/15.6°C).

6.2 pH 1.00 and 2.00 calibrating buffers.

6.3 Feedstock substrate: Feedstock substrate is milled to an appropriate size (normally -3 mm [-1/8 in.]). To prevent possible charring in the reactors, the substrate is often further sieved to remove -60 mesh particles. Upon preparation, the moisture content is determined, following Laboratory Analytical Procedure #001, Standard Method for Determination of Total Solids in Biomass.

6.4 Two 3- to 5-gal pails (required only if the 2-gal system is used) or two 1-L beakers (required only if the 1-L system is used).

6.5 A sink suitable for an ice bath to be prepared for cooling the 2-gal reactor vessel after a pretreatment run (required only if the 2-gal system is used) or an 8-L plastic bucket (required only if the 1-L system is used).

6.6 Two 50-mL graduated cylinders (required only if the 2-gal system is used) or a 10-mL and a 50-mL graduated cylinder (required only if the 1-L system is used).

6.7 pH paper covering pH range 1 to 2. (**Note: If the operator prefers using pH meter to pH paper, this paragraph can be skipped.**)

6.8 Prepare the following four items only if separation of prehydrolyzate and pretreated solids or washing of pretreated solids is required.

6.8.1 A Buchner funnel of 6-L capacity (required only if the 2-gal system is used) or 600-mL capacity (required only if the 1-L system is used).

6.8.2 A 2-L vacuum flask and an aspirator (required only if the 1-L system is used).

6.8.3 A filter cloth (a white cotton bed sheet can be cut into size for use) of approximately 3 ft x 3 ft (required only if the 2-gal system is used) or a Whatman No. 5 filter paper (required only if the 1-L system is used).

6.8.4 pH paper covering pH range 2 to 6. (**Note: If the operator prefers using pH meter to pH paper, this paragraph can be skipped.**)

7. ES&H Considerations and Hazards

7.1 Before any work proceeds, review the appropriate SOP on pretreatment system. It is required that new personnel be trained by an experienced personnel prior to conducting any pretreatment experiment.

7.2 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

7.3 72% H_2SO_4 is very corrosive and must be handled carefully.

7.4 Apparatus in this procedure are heated to elevated temperatures, use caution to avoid burns.

7.5 High pressure is generated in the reactor. Take precautions to avoid unexpected venting of the reactor contents.

8. Procedure for Using 2-gallon Parr Reactor System

8.1 Feedstock Substrate and Chemicals Preparation and Other Preparative Work

8.1.1 Weigh an appropriate amount (approximately 480 g dry weight) of feedstock substrate in a 3- to 5-gal pail. Refer to Paragraph 10.1.1 for an example of the feedstock substrate weight.

8.1.2 Targeting a 10% solids level in the reactor, use a 50-mL graduated cylinder to weigh an appropriate amount of 72% sulfuric acid. The weight of the acid is determined according to calculations shown in Paragraph 10.1.2. (**Note: The target acid level is to obtain a prehydrolyzate pH of 1.3-1.5. Since the prehydrolyzate pH is not known prior to the run, the acid concentration to use has to be preestimated. NREL experience has shown that, for a hardwood substrate, 0.73 wt% acid in the liquid phase in the reactor is generally acceptable. For a herbaceous substrate, 0.88 wt% acid is generally acceptable. Following pretreatment, the prehydrolyzate pH should be tested to determine if the acid concentration used is acceptable. If not so, it must be adjusted and the run repeated until the target pH is met.**)

8.1.3 Targeting at the solids level in the reactor specified above, weigh an appropriate amount of deionized water in the second 3- to 5-gal pail. The weight is determined according to calculations shown in Paragraph 10.1.3.

8.1.4 Thoroughly flush the HPLC pump intake and delivery lines with deionized water (not the water prepared in Step 8.1.3) and discard the effluent. Following flushing, turn off the pump.

8.1.5 Prepare 15 mL deionized water in the second 50-mL graduated cylinder.

8.1.6 Place the pump intake tubing in the acid from Step 8.1.2 and run the pump for approximately 5 minutes at 6 mL/min, directing the effluent from the delivery line back into the acid graduated cylinder. Turn off the pump at the end of the 5-minute period.

8.1.7 Quantitatively transfer the weighed feedstock substrate and water, respectively, from Steps 8.1.1 and 8.1.3 into the reactor (using some of the water to rinse the feedstock substrate pail).

8.1.8 Manually mix the reactor contents.

8.1.9 Close the vent and the chemical injection valves on the reactor head plate, followed by closing the head plate.

8.1.10 After the reactor is securely closed, open the vent valve.

8.2 Pretreatment Run

8.2.1 Use the hoist to load the reactor into the heater, making sure the vent line and the rapture disc relief line on the head plate are facing away from the operator.

8.2.2 Insert thermocouple in thermowell and attach the drive unit.

8.2.3 Turn on the mixer motor, setting the rpm to be about 220 ± 20 (use tachometer to verify rpm). Allow reactor contents to mix at ambient temperature for 5 minutes. Proceed to complete the next step during the 5-minute mixing time.

8.2.4 While Step 8.2.3 is in progress, attach the HPLC pump delivery line to the reactor chemical injection valve.

8.2.5 Following the 5-minute mixing time in Step 8.2.3, turn on the heater and set the temperature controller set-point to 50°C.

8.2.10 As soon as the reactor temperature reaches 159.5°C, turn on the HPLC pump at a flow rate of 6 mL/min, start the stopwatch, followed by opening the chemical injection valve. Monitor time and reactor temperature throughout this period. Prepare a control chart of the reactor pressure at 160°C. The temperature needs to be within 160 " 1°C or the experiment must be repeated.

8.2.11 When the acid graduated cylinder is almost empty (after approximately 4 minutes for a hardwood substrate or 5 minutes for a herbaceous substrate), note the elapsed time and add the deionized water from Step 8.1.5 slowly (to prevent air bubbles from entering the pump intake line) to the acid graduated cylinder to flush out acid in the line to the reactor.

8.2.12 As soon as the deionized water in the acid graduated cylinder has all entered the intake tubing, turn off the pump, close the chemical injection valve, and disconnect the pump delivery line from the valve.

8.2.13 At about the 11.5-minute mark for a hardwood substrate (or 12-minute mark for a herbaceous substrate), turn off the mixer motor, disconnect the drive unit, and move the drive unit and the thermocouple out of way.

8.2.14 Use the hoist to lift the reactor out of the heater and, when the elapsed time reaches 10 minutes plus half of the acid injection time noted in Step 8.2.11, lower the reactor into the ice bath to quench the reaction.

8.2.15 Rotate the mixer shaft manually to assist in cooling uniformly the reactor contents.

8.2.16 When the pressure in the reactor drops to below 5 psig, check with the thermocouple that was removed from the thermowell in Step 8.2.13 to ensure the temperature in the reactor is 95°C or lower. Then, open the vent slowly to relieve the remaining pressure in the reactor.

8.2.17 Upon fully relieving the pressure in the reactor, use the hoist to lift the reactor out of the ice bath and set it upright on the flat surface next to the sink.

8.2.18 Remove the reactor head plate, making sure little or no solids are attached to the mixing unit.

8.2.19 (This step can be skipped if the feedstock substrate has previously been used and the appropriate amount of acid to use is known.) Determine the pH of the prehydrolyzate with the pH meter (the meter should be calibrated with the pH 1.00 and 2.00 calibrating buffers). If the pH is not between 1.3-1.5, repeat the run using an adjusted amount of 72% sulfuric acid. Otherwise, proceed to the next step.

8.2.20 If it is required to separate prehydrolyzate and pretreated solids or to wash the pretreated solids, proceed to Step 8.3. Otherwise, transfer the reactor contents to an appropriate container and label the container properly for storage in a refrigerated area.

8.3 Post-Pretreatment Sample Handling

8.3.1 Set the 6-L Buchner funnel on a clean, dry 3- to 5-gal pail on the floor.

8.3.2 Place the filter cloth over the funnel.

8.3.3 Filter the contents of the reactor through the funnel. Catch the prehydrolyzate in the pail and squeeze the residual prehydrolyzate off the pretreated solids.

8.3.4 Store the prehydrolyzate, sealed and properly labeled, in a refrigerated area.

8.3.5 If washing of the pretreated solids is required, proceed to the next step. Otherwise, store the pretreated solids, sealed and properly labeled, in an appropriate container in a refrigerated area.

8.3.6 Add deionized water to the Buchner funnel to wash the pretreated solids and monitor the pH of the filtrate using pH paper until it reaches the desired pH. Mix the solids with washing water occasionally to ensure effective washing.

8.3.7 Squeeze the residual water off the pretreated solids.

8.3.8 Store the washed pretreated solids, sealed and properly labeled, in an appropriate container in a refrigerated or frozen area.

9. Procedure for Using 1-L Parr Reactor System

9.1 Feedstock Substrate and Chemicals Preparation and Other Preparative Work

9.1.1 Weigh an appropriate amount (approximately 60 g dry weight) of feedstock substrate in a 1-L beaker. Refer to Paragraph 10.2.1 for an example of calculating the feedstock substrate weight.

9.1.2 Targeting a 10% solids level in the reactor, use the 10-mL graduated cylinder to weigh an appropriate amount of 72% sulfuric acid. The weight of the acid is determined according to calculations shown in Paragraph 10.2.2. (**Note: The target acid level is to obtain a prehydrolyzate pH of 1.3-1.5. Since the prehydrolyzate pH is not known prior to the run, the acid concentration to use has to be preestimated. NREL experience has shown that, for a hardwood substrate, 0.73 wt% acid in the liquid phase in the reactor is generally acceptable. For a herbaceous substrate, 0.88 wt% acid is generally acceptable. Following pretreatment, the prehydrolyzate pH should be tested to determine if the acid concentration used is acceptable. If not so, it must be adjusted and the run repeated until the target pH is met.**)

9.1.3 Targeting at the solids level in the reactor specified above, weigh an appropriate amount of deionized water in the second 1-L beaker. The weight is determined according to calculations shown in Paragraph 10.2.3.

9.1.4 Thoroughly flush the HPLC pump intake and delivery lines with deionized water (not the water prepared in Step 9.1.3) and discard the effluent. Following flushing, turn off the pump.

9.1.5 Prepare 15 mL deionized water in the 50-mL graduated cylinder.

9.1.6 Place the pump intake tubing in the acid from Step 9.1.2 and run the pump for approximately 5 minutes at 6 mL/min, directing the effluent from the delivery line back into the acid graduated cylinder. Turn off the pump at the end of the 5-minute period.

9.1.7 Quantitatively transfer the weighed feedstock substrate and water, respectively, from Steps 9.1.1 and 9.1.3 into the reactor (using some of the water to rinse the feedstock substrate beaker).

9.1.8 Manually mix the reactor contents.

9.1.9 Close the vent and the chemical injection valves on the reactor head plate, followed by closing the head plate.

9.1.10 After the reactor is securely closed, open the vent valve.

9.2 Pretreatment Run

9.2.1 Place the reactor into the heater, making sure the vent line and the rupture disc relief line on the head plate are facing away from the operator.

9.2.2 Insert thermocouple in thermowell, attach the drive unit, connect mixer shaft seal cooling water lines, and allow cooling water to flow through the lines (with discharge line directed to the drain).

9.2.3 Turn on the mixer motor, setting the rpm to be about 175 ± 20 (use tachometer to verify rpm). Allow reactor contents to mix at ambient temperature for 5 minutes. Proceed to complete the next step during the 5-minute mixing time.

9.2.4 While Step 9.2.3 is in progress, attach the HPLC pump delivery line to the reactor chemical injection valve.

9.2.5 Following the 5-minute mixing time in Step 9.2.3, turn on the heater and set the temperature controller set-point to 50°C.

9.2.6 The heater will be turned off automatically when the reactor temperature reaches 50°C, but the temperature will continue to rise to about 75°C. Allow the reactor to be heated at about 75°C and mixed for 10 minutes to remove trapped air.

9.2.7 At the end of the 10-minute period, close the reactor vent and adjust the controller set-point to 150°C.

9.2.8 While the reactor is being heated and before the temperature reaches 158°C, prepare an ice bath in the 8-L plastic bucket. (It takes about 20 minutes for the reactor temperature to reach 158°C.)

9.2.9 When the reactor temperature reaches 158°C, change the controller set-point to 160°C.

9.2.10 As soon as the reactor temperature reaches 159.5°C, turn on the HPLC pump at a flow rate of 6 mL/min, start the stopwatch, followed by opening the chemical injection valve. Monitor time and reactor temperature throughout this period. Prepare a control chart of the reactor pressure at 160°C. The temperature needs to be within 160 " 1°C or the experiment must be repeated.

9.2.11 When the acid graduated cylinder is almost empty, note the elapsed time and add the deionized water from Step 9.1.5 slowly (to prevent air bubbles from entering the pump intake line) to the acid graduated cylinder to flush out acid in the line to the reactor.

9.2.12 As soon as the deionized water in the acid graduated cylinder has all entered the intake tubing, turn off the pump, close the chemical injection valve, and disconnect the pump delivery line from the valve.

9.2.13 At about the 9.5-minute mark, turn off the mixer motor, disconnect the drive unit, detach the cooling water lines of the mixer shaft seal, and move the drive unit and the thermocouple out of way.

9.2.14 Lift the reactor out of the heater and, when the elapsed time reaches 10 minutes plus half of the acid injection time noted in Step 9.2.11, lower the reactor into the ice bath to quench the reaction.

9.2.15 Rotate the mixer shaft manually to assist in cooling uniformly the reactor contents.

9.2.16 When the pressure in the reactor drops to below 5 psig, check with the thermocouple that was removed from the thermowell in Step 9.2.13 to ensure the temperature in the reactor is 95°C or lower. Then, open the vent slowly to relieve the remaining pressure in the reactor.

9.2.17 Upon fully relieving the pressure in the reactor, lift the reactor out of the ice bath and set it upright on the bench.

9.2.18 Remove the reactor head plate, making sure little or no solids are attached to the mixing unit.

9.2.19 (This step can be skipped if the feedstock substrate has previously been used and the appropriate amount of acid to use is known.) Determine the pH of the prehydrolyzate with the pH meter (the meter should be calibrated with the pH 1.00 and 2.00 calibrating buffers). If the pH is not between 1.3-1.5, repeat the run using an adjusted amount of 72% sulfuric acid. Otherwise, proceed to the next step.

9.2.20 If it is required to separate prehydrolyzate and pretreated solids, or, to wash the pretreated solids, proceed to Step 9.3. Otherwise, transfer the reactor contents to an appropriate container and label the container properly for storage in a refrigerated area.

9.3 Post-Pretreatment Sample Handling

9.3.1 Set the 600-mL Buchner funnel on the 2-L vacuum flask and connect the aspiration line.

9.3.2 Place a sheet of Whatman No. 5 filter paper in the funnel.

9.3.3 Apply vacuum to filter the contents of the reactor through the funnel. Catch the prehydrolyzate in the vacuum flask. Wait until dripping of prehydrolyzate stops.

9.3.4 Store the prehydrolyzate, sealed (in a bottle, for example) and properly labeled, in a refrigerated area.

9.3.5 If washing of the pretreated solids is required, proceed to the next step. Otherwise, store the pretreated solids, sealed and properly labeled, in an appropriate container in a refrigerated area.

9.3.6 Apply deionized water to the Buchner funnel to wash the pretreated solids and monitor the pH of the filtrate using pH paper until it reaches the desired pH. Mix the solids with washing water occasionally to ensure effective washing.

9.3.7 Wait until dripping of prehydrolyzate stops.

9.3.8 Store the washed pretreated solids, sealed and properly labeled, in an appropriate container in a refrigerated or frozen area.

10. Calculations

10.1 Example calculations for the 2-gallon Parr reactor system:

10.1.1 Weight of feedstock substrate: assuming the moisture content of the feedstock substrate to be 22.0%, then a convenient substrate weight of 615 g can be used. (The dry weight of the substrate is 615 x (1-22.0%) = 480 g.)

10.1.2 Weight of 72% sulfuric acid: since it is known that the target slurry solids level is 10% and the target acid concentration in the reactor liquid phase is 0.73 wt% (assuming the feedstock to be a hardwood), using the numbers from Paragraph 10.1.1, the weight of 72% sulfuric acid is obtained as follows:

$$\frac{615\ x\,(1-22\%)}{10\%}\ x\,(1-10\%)\ x\,0.73\%\ x\ \frac{1}{72\%} = 43.8\ g$$

10.1.3 Weight of dilution water: to obtain a 10% slurry in the reactor using the numbers from Paragraph 10.1.1, the total weight of the liquid phase in the reactor needs to be:

$$\frac{615\ x\,(1-22\%)}{10\%}\ x\,(1-10\%) = 4320\ g$$

$$4320 - 615\ x\ 22\% - 43.8 - 15 = 4120\ g$$

Since the liquid phase (assuming the dilute-acid extractives present in the feedstock substrate to be negligible) in the reactor is comprised of (1) the moisture in the feedstock substrate, (2) the dilution water, (3) the 72% sulfuric acid added, and (4) the acid line flushing water, the weight of the dilution water required is:

10.2 Example calculations for the 2-L Parr reactor system:

10.2.1 Weight of feedstock substrate: assuming the moisture content of the feedstock substrate to be 22.0%, then a convenient substrate weight of 80.0 g can be used. (The dry weight of the substrate is: 80.0 x (1 - 22.0%) = 62.4 g.)

10.2.2 Weight of 72% sulfuric acid: since it is known that the target slurry solids level is 10% and the target acid concentration in the reactor liquid phase is 0.73 wt% (assuming the feedstock to be a hardwood), using the numbers from Paragraph 10.2.1, the weight of 72% sulfuric acid is obtained as follows:

$$\frac{80.0\ x\ (1-22\%)}{10\%}\ x\ (1-10\%)\ x\ 0.73\%\ x\ \frac{1}{72\%} = 5.69\ g$$

$$\frac{80.0\ x\ (1-22\%)}{10\%}\ x\ (1-10\%) = 562\ g$$

10.2.3 Weight of dilution water: to obtain a 10% slurry in the reactor using the numbers from Paragraph 10.2.1, the total weight of the liquid phase in the reactor needs to be:

Since the liquid phase (assuming the dilute-acid extractives present in the feedstock substrate to be negligible) in the reactor is comprised of (1) the moisture in the feedstock substrate, (2) the dilution water, (3) the 72% sulfuric acid added, and (4) the acid line flushing water, the weight of the dilution water required is:

$$562 - 80.0\ x\ 22\% - 5.7 - 15 = 523\ g$$

11. Precision and Bias

11.1 The precision of Parr reactor operation relies largely on operators strictly adhering to the procedure described above. It also relies on the stable performance of instruments, including balance(s), pH meter, pressure gauge, stopwatch, tachometer, and thermocouple (with display) used in the operation. In addition, the stable performance of the HPLC pump used for acid injection affects the precision of the operation. Thus, in addition to the operator's quality skills that must be ensured, the instruments and equipment involved must be maintained in reliable working conditions.

Statistical analysis of a limited database of six replicate runs (using the same substrate and operating conditions by six different operators) shows that the coefficient of variation for the contents of the major components in the pretreated solids (glucan and Klason lignin) and in the prehydrolyzate (glucose, xylose, and acid-soluble lignin) were all below 10%. The coefficient of variation for enzyme digestibility of the pretreated solids was also below 10%.

11.2 Systematic errors may be incurred in preparing dilute-acid pretreated biomass if any of the instruments used is not properly calibrated. It is thus of critical importance to ensure proper calibration of the instruments. Periodic calibration of the various instruments should be performed in accordance with the time intervals recommended by the instrument manufacturers.

12. Quality Control

12.1 *Reported significant figures:* Report prehydrolyzate pH to two decimal places, if using a pH meter and one decimal place if using pH paper. Monitoring of temperature to 0.1°C during Parr reactor runs is required. All weight determinations should include three significant digits.

12.2 *Replicates:* During normal operations, only properly trained operators will be involved and only single runs are required. For QA/QC verification or for training purposes, multiple runs using the same feedstock substrate and the same operating conditions are required either by the same operator or by different operators. The compositions of prehydrolyzate and pretreated solids and/or the digestibility or fermentability of the pretreated solids will then be compared and statistically analyzed to ensure consistency.

12.3 *Blank:* Not applicable

12.4 *Relative percent difference criteria:* Not applicable for single runs. When duplicate runs are conducted, the relative percent difference for glucose, xylose, and Klason lignin of prehydrolyzate and pretreated solids (where applicable) should be no greater than 5%. For acid-soluble lignin, ash, digestibility, and fermentability, the relative percent difference should be no greater than 10%.

12.5 *Method verification standard:* Not applicable

12.6 *Calibration verification standard:* Not applicable

12.7 *Sample size:* Typically, 1-L and 2-gal Parr reactor runs, respectively, use 60 and 480 g dry weight of the prepared feedstock substrate.

12.8 *Sample storage:*

12.8.1 Feedstock: Depending on total solids level and length of storage, the feedstock substrate is recommended to be stored in ambient conditions (when total solids content is above 88% and, thus, no risk of deterioration is involved) or refrigerated (when total solids content is below 88% for up to 1 month storage) or frozen (when total solids content is below 88% for prolonged storage).

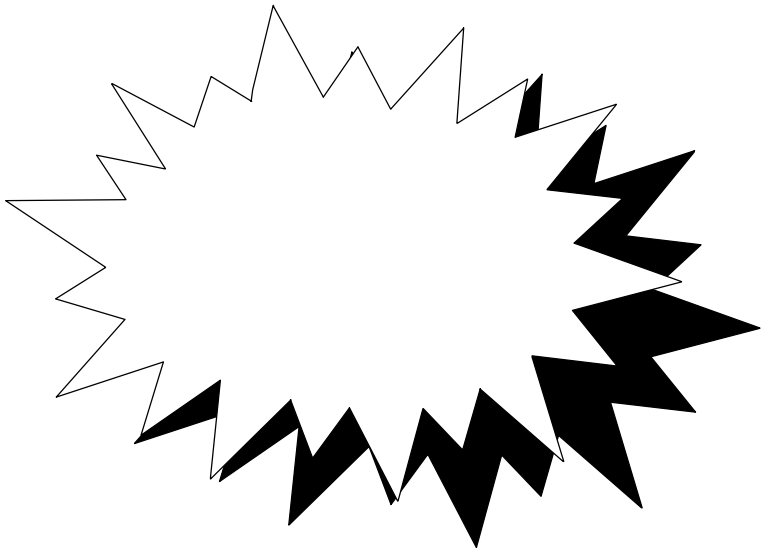
12.8.2 Pretreated products: Prehydrolyzate or pretreated solids samples should be refrigerated. It is further recommended to store pretreated solids in acidified conditions. If washing and prolonged storage of a washed pretreated solids sample is required, freezing is recommended.

12.8.3 *Standard storage:* Not applicable

12.8.4 *Standard preparation:* Not applicable

12.8.5 *Definition of a batch*: Not applicable

12.8.6 *Control charts:* Prepare a control chart of reactor pressure at 160EC for each pretreatment run.



Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-008

Procedure Title: SSF Experimental Protocols: Lignocellulosic Biomass Hydrolysis and Fermentation

Author(s): Tammy Kay Hayward, Nancy S. Combs, Sherry L. Schmidt, George P. Philippidis

Date: 5/31/95

ISSUE DATE: 5-31-95 **SUPERSEDES:** 6/4/93

SSF Experimental Protocols:
Lignocellulosic Biomass Hydrolysis and Fermentation

Laboratory Analytical Procedure #008

1. Introduction

1.1 Ethanol is a promising alternative fuel which can be produced biologically from a variety of waste materials such as paper products, corn fiber, sawmill waste, straw, and rice. Ethanol has been made from grapes, barley and potatoes for thousands of years. The production of ethanol from non-starch, lignocellulosic materials is, however, a fairly recent development. There are many ways to produce ethanol from lignocellulosic material. The method discussed here is known as simultaneous saccharification and fermentation (SSF). It utilizes cellulase enzyme to break down the cellulose and yeast to ferment the resulting glucose. The ethanol can be blended with gasoline or used neat in combustion engines. As a fuel, ethanol burns cleaner than gasoline, is completely renewable, and relatively less toxic to the environment.

2. Scope

2.1 The described protocols have been developed based on the personal experience of NREL researchers with biomass conversion and may be revised periodically. It is the sole responsibility of the user of the protocols to obtain updated versions from the NREL technical monitor. These procedures and their revisions **by no means** represent optimal conditions for the described experimentation and are proposed simply as a means of maintaining consistency. Furthermore, the results may vary depending on the expertise of the researcher and the quality of the materials employed in the studies.

2.2 This LAP consists of two separate sub-procedures. The first is "Hydrolysis of Lignocellulosic Biomass". The second is "Simultaneous Saccharification and Fermentation of Biomass". This procedure is intended to test a variety of lignocellulosic substrates and provide a consistent method for their evaluation among NREL subcontractors. **The procedures are intended for raw biomass substrates or washed, pretreated substrates only** i.e. pretreated substrates containing acetic acid, furfural, and/or other inhibitors of yeast metabolism must be extensively washed with water to remove these inhibitors prior to the experiments.

2.3 All analyses shall be performed according to the Ethanol Project Quality Assurance Plan (QAP).

3. References

3.1 NREL Ethanol Project CAT Task Laboratory Analytical Procedure #001, "Standard Method for Determination of Total Solids in Biomass".

3.2 NREL Ethanol Project CAT Task Laboratory Analytical Procedure #002, "Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography".

3.3 NREL Ethanol Project CAT Task Laboratory Analytical Procedure #006 "Measurement of Cellulase Activities".

3.4 NREL Ethanol Project CAT Task Laboratory Analytical Procedure #011, "Determination of Ethanol Concentration in Biomass to Ethanol Fermentation Supernatants by Gas Chromatography".

3.5 NREL Ethanol Project CAT Task Laboratory Analytical Procedure #013, "HPLC Analysis of Liquid Fractions of Process Samples for Soluble Sugars".

3.6 NREL Ethanol Project CAT Task Laboratory Analytical Procedure #015, "HPLC Analysis of the Liquid Fractions of Process Samples for Organic Acids, Glycerol, HMF, and Furfural".

3.7 T. Vinzant, L. Ponfick, N. Nagle, C. Ehrman, J. Reynolds, and M. Himmel, "SSF Comparison of Selected Woods from Southern Sawmills." 1994. Applied Biochemistry and Biotechnology Vol. 45/46 pp 611-626.

3.8 G. Philippidis, T.K. Smith and C. Wyman, "Study of the Enzymatic Hydrolysis of Cellulose for Production of Fuel Ethanol by Simultaneous Saccharification and Fermentation Process." 1993. Biotechnology and Bioengineering Vol. 41 pg 846-853.

4. Terminology

4.1 Saccharification (SAC) or hydrolysis of lignocellulosic biomass: the addition of enzyme to lignocellulosic biomass which results in the formation of oligomers, cellobiose and glucose. It is performed under sterile conditions and is also referred to as digestibility.

4.2 Simultaneous saccharification and fermentation (SSF): a method for producing ethanol from lignocellulosic biomass in which both enzymatic saccharification of cellulose by enzymes and fermentation of the resulting sugars by yeast occur at the same time in the same vessel.

5. Apparatus

5.1 In addition to the equipment described in LAPs 01, 02, 06, 011, 013, and 015 the following are required for this work.

5.2 An **autoclave** is necessary for the sterilization of media and flasks both prior to and after experiments.

5.3 A **laminar flow hood or biosafety cabinet** is necessary for sterile sampling.

5.4 A **-70^{o}C freezer** is necessary for the storage of frozen yeast cultures.

5.5 A bench top **centrifuge** is required for SSF sample preparation.

5.6 A **shaker incubator** is necessary for the SSF's in order to keep the fermentations at 38^{o}C +/- 2^{o}C and 150 rpm.

5.7 **Bubble traps**, also called gas locks, CO_2 traps and water traps, are devices which prevent air from entering the shake flask and at the same time allow carbon dioxide to escape. They must be autoclavable. One such device is a rubber stopper through which a glass tube is inserted. A cotton plug is placed in the tube and the tube is connected to silicone tubing the end of which is submerged in a test tube with H_2O. The test tube is taped to the side of the flask. Another device that can be inserted into a rubber stopper is all glass and has a u-tube filled with water. The carbon dioxide can bubble out, but the water prevents the air from entering. SSF's require a bubble trap.

5.8 An **analytical balance** is necessary for accurately measuring out biomass samples and preparing SSF flasks.

5.9 **Cell counting chamber slide** (for ex. hemocytometer) for yeast cell counts.

5.10 **Microscope** capable of 1000 times magnification.

5.11 Autoclavable **shake flasks**, **Morton closures** (metal caps), and sterile **pipets** (disposable with tips that can be broken off conveniently to provide the wide opening needed for sampling SSF slurries).

5.12 **Convection oven**, with temperature control of 80 ± 3°C, **desiccator**, and **aluminum foil weighing dishes** for dry cell mass concentration measurements.

5.13 A **glucose analyzer** is suggested for rapid analysis of glucose. Manufacturers include Yellow Springs Instruments.

6. Solutions, Media, and Stock Cultures

6.14 10X YP medium (liquid)

Yeast extract	100 g/L
Peptone	200 g/L

Adjust pH to 5.0 with sulfuric acid. Autoclave for 30 minutes at 121^{o}C.

Yeast Extract, Peptone, Dextrose (YPD) media is a common growth medium for yeast. It is rich in amino acids, vitamins, and minerals necessary for yeast growth and fermentation. This complex medium is supplied in excess, so that nutrients are not a limiting factor. Although this yeast will grow at other pH conditions, pH 5 is chosen because it is optimal compromise for SSF of most substrates when using common cellulases.

6.15 YPD Plates (solid medium)

Yeast extract	10 g/L
Peptone	20 g/L
Dextrose (glucose)	20 g/L
Agar	15 g/L

Dissolve dextrose in deionized (DI) water. Weigh agar into container, add glucose solution. Autoclave for 30 minutes at 121^{o}C. Let cool and add sterile 10X YP medium. Mix solution gently, and aseptically pour the plates. Store plates inverted in the refrigerator.

6.16 YPD medium (liquid)

Yeast extract	10 g/L
Peptone	20 g/L
Dextrose (glucose)	20 g/L

Adjust pH to 5.0 with sulfuric acid and then filter sterilize (do not autoclave glucose and YP together.)

6.17 Antibiotics

If desired, use 0.2 mL of penicillin and/or 0.2 mL of streptomycin filter sterilized stock solutions in the SSF mixture (stock solutions: Penicillin 5 g/L; Streptomycin 5 g/L). Use of antibiotics is not recommended because of the added ES&H risks. (See ES&H section)

6.18 Frozen stock culture of *Saccharomyces cerevisiae* D_5A

50 mL sterile 40% glycerol
50 mL inoculum from NREL-supplied plate

Autoclave or filter sterilize a 40% solution of glycerol in DI water. Let cool to room temperature. Prepare initial inoculum from the plate by transferring culture into 100 mL of YPD media in a sterile 250 mL flask. Incubate in a rotary shaker at 38^{o}C for 24 hours. Test for pH, glucose, and ethanol. The pH should be between 4.5 and 5.0, glucose should be between 0 and 5 g/L, and ethanol between 8 and 10 g/L. Observe the culture under the microscope for bacterial contamination and culture purity. Mix the glycerol and inoculum aseptically. Dispense one milliliter aliquots into sterile cryovials. Place in a -70^{o}C freezer. Each cryovial will have a standardized number of yeast cells per vial and subsequent SSF inocula should be prepared using a frozen vial..

Once every three months perform a viability check on the frozen stock. Thaw one vial. Vortex to resuspend cells. Perform a cell count with a hemacytometer under the microscope. Then, perform colony forming unit (CFU) tests using YPD plates by diluting the culture with sterile saline (0.89% NaCl solution) to obtain a spread plate cell count of 30-300 cells/plate.

The number of cells on the plate multiplied by the dilution factor gives you the CFU's/mL.
Percent viability is CFUs/mL divided by the hemacytometer count/mL. For example 1.0×10^7 CFU/mL and 1.0×10^8 cells counted/mL gives a viability of 10%. Make a new frozen stock when the % viability drops below 50%. Maintain a control chart on the viability.

6.19 Liquid cellulase enzyme preparation.

Filter sterilize all enzyme upon arrival. Use non-cellulosic based filters such as the 0.45 mm VacuCap 90 from Gelman, product number 4624 made of polyethersulfone. Nylon and glass pre-filters are also suggested. Store enzyme in the refrigerator in sterile containers. The activity should be monitored using the LAP-006, "Measurement of Cellulase Activities". Cellulase activity values should be used to track enzyme

stability over time. A control chart should be created for this purpose. However, the SSF and SAC loadings need to be based on a consistent and standardized number, that being the official NREL Filter Paper Units per milliliter number for that preparation. This standardization allows us to more easily compare SSF and SAC data obtained from different subcontracts.

7. ES&H Considerations and Hazards

6.20 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

6.21 Treat all biological growths with caution. Do not smell flasks as a method of checking for contamination. Any contaminant microorganism has the potential of being a health hazard.

6.22 The use of antibiotics in fermentations can lead to antibiotic resistant microbes which can cause persistent infections in researchers. In addition, prolonged exposure to antibiotics can cause allergic reactions to a variety of medications.

6.23 Avoid breathing dusts of yeast extract by weighing and transferring the solid in a chemical fume hood. Yeast extract dust can coat the lungs and cause allergic reactions and/or breathing problems. Dust masks are also recommended.

6.24 Autoclave all samples from SSF or SAC or inoculum prior to disposal. Treat unautoclaved glassware, etc., with a 1% Chlorox or 30% hydrogen peroxide solution to kill organisms prior to washing.

8. Procedure for the Hydrolysis of Lignocellulosic Biomass (SAC)

6.25 The goal of this procedure is to test pretreated or raw biomass substrates by determining the initial hydrolysis (SAC) rate during saccharification catalyzed by cellulase enzymes. Microorganisms are not employed in this experiment.

6.26 Based on the biomass moisture content (LAP-001) and cellulose content (LAP-002) data, determine the quantity of biomass needed. Shake flasks should have stopper or Morton closure and a 2:5 medium to flask volume ratio. **All SAC flasks need to have a 1% w/w effective cellulose content. Do not dry pretreated substrates;** once dry the pores of the biomass permanently collapse and do not have the same digestibility**.**

Example: 0.5263 g of alpha-cellulose is the weight needed based on the calculation for a 50 gram working weight and 95% total solids. 4.167 grams is the weight needed of a pretreated wood with a 60% cellulose content (LAP-002) and a 20% total solids content (LAP-001) in a final working weight of 50 grams.

6.27 Based on the filter paper activity of the cellulase enzyme (FPU/mL) and the working weight of the flask, determine the volume of enzyme needed. **All flasks need to have an enzyme loading of 25 FPU/g cellulose based on the official NREL value for the cellulase enzyme preparation**. If all flasks in an experiment will use the same lot of enzyme and the same working weight, then the same volume of enzyme (for example 1.5 mL) will be added to each flask.

Enzyme loading is the most critical factor affecting rates and yields. More accurate addition of the enzyme can be obtained if it is diluted. Also, the new, diluted enzyme can be freshly filtered to ensure sterility. Use freshly diluted sterilized enzyme for each experiment to ensure enzyme activity has not decreased and sterility is maintained. Do not store the enzyme in diluted form for over one day.

Example calculation: 1.506 mL of 1:10 diluted enzyme is needed for an experiment where the diluted enzyme solution has an activity of 8.3 FPU/mL and a SAC flask working volume is 50 mL. Prior to starting an experiment, prepare a fresh, filter sterile (non-cellulosic filter) 1:10 dilution of enzyme in 1x media. Dilution is performed so that the volume added can be accurately measured and easily dispersed into the flask.

6.28 Using the substrate weight and diluted enzyme volume determined in the previous steps, calculate the amount of DI water and 10X YP medium needed. An example of a SAC flask recipe is as follows:

4.167 g	pretreated wood (1% w/w cellulose, substrate has a 60% cellulose content and a 20% total solids content)
1.5 mL	1:10 diluted NREL-supplied cellulase enzyme
5.0 mL	10X YP solution
39.33 mL	DI water (50 - 1.5 - 5 - 4.167)
50.0 grams total (working weight)	

6.29 Each experiment should include an alpha-cellulose control substrate. All substrates should be tested in duplicate at a minimum. A control chart should be set up to compile the alpha-cellulose final yields over time.

6.30 Record the actual amount of substrate weighed into each flask to at least the nearest milligram. *For example 0.5321 grams of alpha-cellulose in Flask #1 and 0.5200 grams for Flask #2*. Percent digestibility will be based on this number. Be as actuate as possible and re-calculate the actual enzyme loading.

6.31 Add the DI water and 10X YP media. Gently swirl the flask and completely wet the biomass chips.

6.32 Check and adjust the pH of the slurry of each flask/vessel to 5.0 ± 0.2 with either lime or sulfuric acid. Add stoppers to the flasks. Weigh to the nearest milligram the entire flask set-up and record as pre-autoclave weight.

6.33 Since all the work up to this point has not been done aseptically, autoclave the flasks and/or vessels within two hours. Autoclave at 121^{o}C for 30 minutes (1 hour for a fermentor containing 4 L of medium; make sure the vessel can ventilate freely). Let the flasks/vessels cool to room temperature.

6.34 Reweigh the flasks to the nearest milligram and record the post-autoclave weight. Add back lost weight as sterile mLs of DI water.

6.35 In a laminar flow hood aseptically add to the first flask or vessel, the required volume of filter sterilized cellulase enzyme, as determined in step 8.3.

6.36 Mix the flasks by swirling. Aseptically take a time zero slurry sample. (see step 8.15).

6.37 Incubate the flasks in a rotary shaker at 150 rpm and 38^{o}C. For vessels, set the agitation speed at 150 rpm.

6.38 Repeat steps 8.11-8.13 with the other flasks. Start each flask individually and note the time of completion for first and last flasks.

6.39 At appropriate sampling times (for example: 0, 3, 6, 24, 48, 72, and 168 hours) take 3 mL slurry samples aseptically with sterile large mouth pipet tips or pipets for flasks, or through a port of about 0.5" internal diameter for vessels. Store in capped tubes/vials. Place the samples on ice until all the samples of that specific time point have been collected. Place the capped tubes/vials in a boiling water bath for exactly 5 minutes to inactivate the enzyme. Chill on ice.

6.40 Centrifuge and filter to remove denatured enzyme and lignocellulosic biomass. Determine the amount of glucose present in each supernatant sample by YSI or HPLC (LAP-013). Measure the concentration of cellobiose by HPLC (LAP-013) for at least 3 of the time-points. If the analysis will be done later, freeze the supernatant in sealed HPLC glass vials.

6.41 For the **last time point** make samples for YSI and HPLC as in step 8.15-8.16. In addition, streak a sample from each flask or vessel on a YPD plate to check for contamination by any microorganism. Observe, under the microscope, a sample of the slurry for the presence of contaminants. Report the final slurry pH of each flask or vessel.

6.42 All test tubes and flasks containing cultures or samples should be autoclaved prior to disposal. All other items (e.g. pipettes, syringes) that come into contact with the culture should be placed in containers of biocidal solutions before sterilization and reuse or disposal. Contaminated pipette tips and microcentrifuge tubes should be autoclaved before disposal.

6.43 Autoclave the residual hydrolysis material, making sure that the stoppers are loose enough to allow ventilation. Ensure that fermentors can ventilate freely. Sterilized liquids may be discharged down the drain after the solids are removed and thrown in the trash.

6.44 Calculate the glucose yield as % of the theoretical yield (% digestibility) by using the following formula:

$$\%Yield = \frac{[Glucose] + 1.053\,[Cellobiose]}{1.111\ f\ [Biomass]}\ X\ 100\%$$

where:

[*Glucose*]	Residual glucose concentration (g/L)
[*Cellobiose*]	Residual cellobiose concentration (g/L)
[*Biomass*]	Dry biomass concentration at the beginning of the fermentation (g/L)
f	Cellulose fraction in dry biomass (g/g)

The multiplication factor, 1.053, converts cellobiose to equivalent glucose.

6.45 Graph and/or tabulate the collected data (glucose and cellobiose concentration vs. time) for each experiment.

6.46 Quality Control

6.46.1 *Reported significant figures:* Report % digestibility to one decimal place.

6.46.2 *Replicates:* At least duplicate flasks.

6.46.3 *Blank:* None.

6.46.4 *Relative percent difference criteria:* 5% digestibility within one set of flasks run at the same time, by the same person, in the same shaker, with the same analytical instrument.

6.46.5 *Method verification standard:* Alpha-cellulose control. The moisture content of alpha-cellulose must be measured every 3 months.

6.46.6 *Calibration verification standard:* None.

6.46.7 *Sample size:* See flask preparation instructions.

6.46.8 *Sample storage:* Store wet pretreated biomass in the refrigerator for no more than 3 weeks, otherwise freeze it. Store dry (88% or more total solids) biomass at room temperature. Do not use biomass that exhibits signs of spoilage.

6.46.9 *Standard storage:* Alpha-cellulose is considered dry biomass and can be stored at room temperature.

6.46.10 *Standard preparation:* None.

6.46.11 *Definition of a batch*: Flasks started at the same time with one set of alpha-cellulose controls.

6.46.12 *Control charts:* % digestibility, at time final for alpha-cellulose and enzyme activity controls. See 8.22.14.

6.46.13 *Sterility verification:* In all flasks, the final pH should be 5.0 " 0.2, no microbes should be detected by microscope or plate checks. Flasks that did not remain sterile must be repeated.

6.46.14 *Enzyme activity:* The filter paper activity, (LAP-006) FPU/mL, must be measured every 6 months. Keep an activity control chart, but use the official NREL cellulase activity number for enzyme loading calculations.

9. Procedure for Simultaneous Saccharification and Fermentation (SSF)

6.47 Inoculum preparation

6.47.1 The goal of this procedure is to prepare a seed culture for SSF. An aerobic fermentation of glucose is used to produce yeast cell mass.

6.47.2 This inoculum preparation procedure involves two growth stages. The first stage, pre-inoculum, is a flask in which the frozen stock culture, containing a standardized number of cells, is inoculated into YPD (liquid medium). This stage eases the yeast in its transition from stasis to growth phase. The growth

phase occurs in a second flask which contains YPD and is inoculated from the first.

6.47.3 To prepare the first stage flask, transfer 50 mL of sterile YPD into a 125 mL sterile baffled shake flask with a Morton closure (metal cap).

6.47.4 To prepare the second stage flask, transfer the desired amount of YPD into the appropriate sterile Morton closure flask. Base flask size on sufficient inoculum for the SSF experiment (10% v/w transfer) plus at least 20 mL for sample analysis etc. Maintain a 2:5 liquid to flask volume ratio. Account for a 10% v/v seed volume from the preinoculum.

Example: for an experiment containing six 100 g SSFs, 60 mL plus 20 mL is 80 mL needed, then to give a 2:5 ratio, we round off to 100 mL in a 250 mL flask. The inoculum recipe would be as follows: 10 mL of preinoculum into 90 mL of YPD in a sterile 250 mL baffled flask with a Morton closure.

6.47.5 Inoculate the first-stage YPD flask (pre-inoculum) with one thawed stock vial of *Saccharomyces cerevisiae* D_5A. Incubate at 38^{o}C and 150 rpm for 6-8 hours. Before transferring to the next stage, check microscopically for contamination. Only use pure cultures.

6.47.6 Inoculate the second-stage with a 10% v/v transfer from the pre-inoculum. Incubate at 38^{o}C and 150 rpm for 12-16 hours. Before transferring, check microscopically for contamination and analyze for residual glucose concentration. The culture can be transferred once the glucose falls below 2 g/L. Optimally, there should be some residual glucose to ensure cells are still in the growth phase. Check pH and perform DCM (dry cell mass) analysis. Prepare samples for HPLC and GC analysis at the end of the inoculation. Read the ethanol content and use 10% of the value as the time zero ethanol concentration in the SSF flasks. Use only pure D_5A cultures.

6.47.7 Create an inoculum control chart with the final DCM, pH, ethanol, and glucose concentrations for each inoculum.

6.48 Procedure For Dry Cell Mass Concentration

6.48.1 The goal is to measure the dry cell mass concentration.

6.48.2 Dry aluminum dishes in the oven at 80^{o}C overnight.

6.48.3 Cool the dishes in a desiccator for 30 minutes.

6.48.4 Record each dish weight to four decimal places using an analytical balance.

6.48.5 Using a sterile pipet, take a 10-mL inoculum sample, centrifuge, and wash the cell pellet twice with 10 mL of DI water (2 volume wash). After the second wash and centrifuge cycle, resuspend the pellet in 5 mL of DI water.

6.48.6 Transfer pellet by repeated vortex washes with DI water to a weighed dish.

6.48.7 Dry the dishes and cells in the oven at 80^{o}C overnight.

6.48.8 Cool the dishes in the desiccator for 30 minutes.

6.48.9 Record the weight of the dishes plus dried cells.

6.48.10 Calculate the dry cell mass concentration of the inoculum in *g/L* by using the following formula:

$$DCM = \frac{weight\ of\ dish\ plus\ dried\ cells - weight\ of\ dish}{0.01\ L}$$

6.49 Procedure for the Simultaneous Saccharification and Fermentation of Biomass

6.49.1 The goal of this procedure is to assess the conversion of lignocellulosic biomass into ethanol using the SSF process. This procedure is almost identical to the saccharification protocol differing only in the following ways: (1) **yeast** is used to convert the glucose into ethanol, (2) cellulose content is higher at **3% w/w,** (3) **bubble traps** are used to maintain anaerobic conditions and (4) carbon dioxide, ethanol, glycerol, lactic and acetic acid are formed and residual glucose and cellobiose levels remain low.

6.49.2 Determine the amount of biomass needed for each SSF flask based on the biomass moisture and cellulose content. All flasks should have a **3% w/w effective cellulose concentration. Do not dry pretreated substrates.** Once dry, the pores of the biomass permanently collapse. Shake flasks should have a 2:5 medium to flask volume ratio and should be equipped with **water traps**.

6.49.3 Based on the filter paper activity of the cellulase enzyme (FPU/mL) and the working weight of the flask, determine the volume of enzyme needed. All flasks should use an enzyme **loading of 25 FPU/g cellulose using the official NREL FPU/mL for the enzyme preparation**. The volume of enzyme added will be the same for all SSF flasks with the same working weight. Prior to starting an experiment, prepare a fresh, filter sterile (non-cellulosic filter) 1:10 dilution of enzyme in media. Dilution is performed so that the volume added can be accurately measured and easily dispersed into the flask. Do not store the enzyme in diluted form for over one day.

Enzyme loading is the most critical factor affecting rates and yields. More accurate addition of the enzyme can be obtained if it is diluted. Also, the new, diluted enzyme can be freshly filtered to ensure sterility.

6.49.4 Using the above substrate weight and diluted enzyme volume, calculate the amount of DI water and 10X YP medium needed. Below is an example SSF recipe for a 250 mL water-trap flask:

For example:

3.14 g	alpha-cellulose (LAP-001, 95% moisture)
10.0 g	10X YP
10.0 mL	*S. cerevisiae* D_5A inoculum (second stage)
9.04 mL	NREL supplied 1:10 diluted cellulase enzyme assuming an undiluted volumetric activity of 83 FPU/mL
67.82 g	DI water

100.0 grams total	

note: It is easier to add the enzyme and inoculum via a sterile pipet. The density of both ingredients is assumed to be 1 g/L.

6.49.5 Each experiment should include an alpha-cellulose control substrate. All SSF should be performed in duplicate at a minimum. A control chart should be set up to include the ethanol yields from a standard final time point (for example 168 hours) of alpha-cellulose SSF.

6.49.6 Tare the first flask, weigh in substrate and 10X YP media. Record the actual amount of substrate weighed into each flask to at least the nearest milligram. *For example 3.167 grams of alpha-cellulose in Flask #1 and 3.1743 grams for Flask #2.* Percent theoretical ethanol will be based on this number. Be as accurate as possible and re-calculate the actual enzyme loading.

6.49.7 Check and/or adjust the pH of the slurry to 5.0 ± 0.2 with either lime or sulfuric acid. Account for any change in weight by adding less DI water.

6.49.8 Add DI water. Gently swirl flask to completely wet the biomass chips in the liquid.

6.49.9 Add water trap, autoclave tape, label etc. to flask (do not place any water in the traps at this time.) Weigh the whole flask to the nearest milligram assembly and record this weight as pre-autoclave. Repeat for each SSF.

6.49.10 Since all the work up to this point has not been done aseptically, autoclave the flasks and/or vessels as soon as possible. Autoclave at 121^{o}C for 30 minutes (1 hour for a fermentor containing 4 L of medium; make sure the vessel can ventilate freely). Let the flasks/vessels cool to room temperature. Re-weigh each flask assembly to the nearest milligram and add back lost weight as mL of sterile DI water.

6.49.11 In a laminar flow hood aseptically add to the first flask or vessel:

(a) 10% v/w yeast inoculum (in this case 10 mL)
(b) Required mL of filtered sterilized 1:10 diluted cellulase enzyme (in this case 9.04 mL)
(c) Swirl to mix flask ingredients well.

6.49.12 Add water to the water/CO_2 trap of the flask and incubate the flasks in a shaker at 150 rpm and 38^{o}C. For vessels, set the agitation speed at 150 rpm.

6.49.13 Repeat with the other flasks. Start each flask separately and record the time of completion for first and last flasks.

6.49.14 At appropriate sampling times (for example 24, 48, 72, 96, 120, 144, and 168 hours) take 4 mL slurry samples aseptically with sterile large mouth pipet tips or pipets for flasks, or through a port of about 0.5" internal diameter for vessels. Store in capped tubes/vials. Chill on ice, centrifuge, collect and filter the supernatant. Analyze for glucose and cellobiose (LAP-013), glycerol, lactic acid, and acetic acid (LAP-015) by HPLC, and ethanol (LAP-011) by GC. If the analysis will be done later, freeze the filtered supernatant in HPLC/GC glass vials.

6.49.15 For the **last time point** make samples for YSI, GC and HPLC. In addition, streak a sample from each SSF flask or vessel on a YPD plate. Plates should show viable yeast with no contaminant organisms. Observe slurry under the microscope for presence of biomass fibers and yeast cells. The presence of foreign organisms is cause for repeating the SSF. Read and record the slurry pH of each flask or vessel. Final pH should be 5.0 +/- 0.7. Drop in pH to less than 4.0 is cause for repeating the SSF. If possible, perform compositional analysis (LAP-002) of the SSF residue and close mass balance.

6.49.16 All test tubes and flasks containing cultures or samples should be autoclaved prior to disposal. All other items (e.g. pipettes, syringes) that come into contact with the culture should be placed in biocidal solution before washing, reuse and/or disposal.

6.49.17 Autoclave the residual SSF material, making sure that the CO_2 traps are dry, so that the flasks can ventilate. For fermentors, ensure that they can ventilate freely. Sterilized liquids may be discharged down the drain after the solids (biomass, dead yeast cells, and denatured enzyme) are removed and thrown in the trash.

6.49.18 Calculate the ethanol yield as % of the theoretical yield by using the following formula:

$$\%Yield = \frac{[EtOH]_f - [EtOH]_o}{0.568 \; f \; [Biomass]} \; X \; 100\%$$

where:

$[EtOH]_f$	Ethanol concentration at the end of the fermentation (g/L)
$[EtOH]_o$	Ethanol concentration at the beginning of the fermentation (g/L)
[*Biomass*]	Dry biomass concentration at the beginning of the fermentation (g/L)
f	Cellulose fraction of dry biomass (g/g)
0.568	Conversion Factor for cellulose to ethanol based on stoichiometric biochemistry of yeast.

6.49.19 Report, graph, and/or tabulate information about each experiment including:

- Any observations about the experiment (e.g. foaming, color, etc.).
- Any deviations from the standard protocol.
- Residual and produced ethanol concentration, as well as theoretical yield vs. SSF time (expressed in hours.)
- If possible, residual concentration of glycerol and other metabolites.
- Final pH of SSF.
- Contamination assessment and description of the morphology (shape, color, size, and texture) of the colonies appearing on the plates, including *S. cerevisiae* D_5A.

6.50 Quality Control

6.50.1 *Reported significant figures:* Report % theoretical yield to one decimal place.

6.50.2 *Replicates:* At least duplicate flasks.

6.50.3 *Blank:* None.

6.50.4 *Relative percent difference criteria:* 5% yield within one set of flasks run at the same time, by the same person, in the same shaker, with the same analytical instrument.

6.50.5 *Method verification standard:* Sigma alpha-cellulose control. The moisture content of alpha-cellulose needs to be measured every 3 months.

6.50.6 *Calibration verification standard:* None.

6.50.7 *Sample size:* Not applicable.

6.50.8 *Sample storage:* Store wet pretreated biomass in the refrigerator for no more than 3 weeks, otherwise freeze it. Store dry (88% or more total solids) biomass at room temperature. Do not use biomass that exhibits signs of spoilage.

6.50.9 *Standard storage:* Sigma alpha-cellulose can be stored at room temperature.

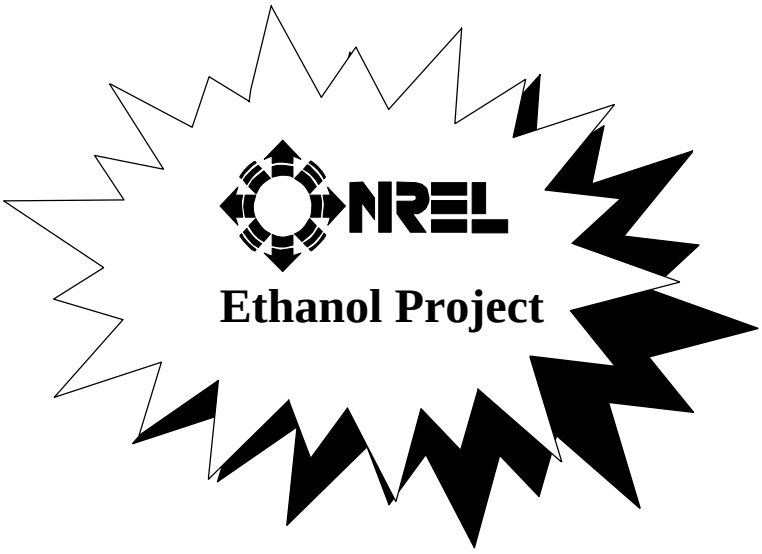
6.50.10 *Standard preparation:* None.

6.50.11 *Definition of a batch*: Flasks started at the same time by the same researcher.

6.50.12 *Control charts:* Make a control chart of alpha-cellulose SSF results (final-day ethanol (g/L), final pH, and % theoretical yield) for each experiment. Make inoculum control chart, enzyme activity control chart, frozen stock viability control chart.

6.50.13 *Other:* Verification of a pure D_5A culture--all flasks--final pH should be 5.0 +/- 0.7, no microbes other than D_5A should be detected by microscope or plate checks. Contaminated flasks need to be repeated.

6.50.14 *Enzyme activity:* Measure enzyme activity using LAP-006, (Measurement of Cellulase Activities) every 6 months. Tabulate and make a control chart. Again, use the official NREL number for the SSF. Enzyme loading is the most critical factor in rates and yields of ethanol production via SSF.



Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-009

Procedure Title: Enzymatic Saccharification of Lignocellulosic Biomass

Author: Larry Brown and Robert Torget

Date: 8/19/96

ISSUE DATE: 8/26/96 **SUPERSEDES:** 8/11/93

Enzymatic Saccharification of Lignocellulosic Biomass

Laboratory Analytical Procedure #009

1. Introduction

1.1 This procedure describes the enzymatic saccharification of cellulose from native or pretreated lignocellulosic biomass to glucose in order to determine the maximum extent of digestibility possible (a saturating level of a commercially available or in house produced cellulase preparation and hydrolysis times up to one week are used).

2. Scope

2.1 This procedure is appropriate for lignocellulosic biomass. If the biomass is suspected to have some starch content, dry weight percent cellulose calculated from total glucan (LAP-002) must be corrected to subtract the starch contribution to total dry weight percent glucose.

2.2 All analyses shall be performed according to the guidelines established in the Ethanol Project Quality Assurance Plan (QAP).

3. References

3.1 Grohmann, K., Torget, R., and Himmel, M. (1986), Biotech. Bioeng. Symp. No. 17, 135-151.

3.2 Ghose, T.K. (1987), Pure & Appl. Chem., **59,** 257-268.

3.3 Stockton, B.C., Mitchell, D.J., Grohmann, K., and Himmel, M.E. (1991), Biotech. Let., **13,** 57-62.

3.4 Adney, B. and Baker, J. (1993), Ethanol Project Laboratory Analytical Procedures, LAP-006, National Renewable Energy Laboratory, Golden, CO, 80401.

3.5 Ehrman, C. I. (1996), Ethnaol Project Laboratory Analytical Procedures, LAP-016, National Renewable Energy Laboratory, Golden, CO, 80401.

4. Terminology

4.1 Pretreated biomass - Biomass that has been subjected to milling, chemical treatment with water or steam, strong or dilute acid or alkali, or other physical or chemical methods to render the cellulose content of the material more accessible to enzymatic action.

4.2 Cellulase enzyme - an enzyme preparation exhibiting all three synergistic cellulolytic activities: endo-1,4-β-D-glucanase, exo-1,4-β-glucosidase, or β-D-glucosidase activities, which are present to different extents in different cellulase preparations.

5. Significance and Use

5.1 The maximum extent of digestibility is used in conjunction with other assays to determine the appropriate enzyme loading for saccharification of biomass.

6. Interferences

6.1 Test specimens not suitable for analysis by this procedure include acid- and alkaline-pretreated biomass samples that have not been washed. Unwashed pretreated biomass samples containing free acid or alkali may change solution pH to values outside the range of enzymatic activity.

7. Apparatus

7.1 VWR model 1540 incubator set at $50^{o} \pm 1^{o}C$.

7.2 Cole-Parmer model 7637-20 "Roto-Torque" Fixed Speed Rotator.

7.3 A 24-slot large-holed test tube rack that can be attached to the "Roto-Torque" Rotator.

7.4 Eppendorf model 5414 microcentrifuge.

7.5 pH meter.

7.6 Analytical balance, sensitive to 0.0001 g.

7.7 Yellow Springs Instrument, Inc., Model 27 Glucose Analyzer or Model 2700 Select Biochemistry Analyzer.

7.8 Drying oven adjusted to $105^{o}C \pm 2^{o}C$.

7.9 A 200 µL and a 1000 µL Eppendorf Pipetman pipet with tips.

8. Reagents and Materials

8.1 Tetracycline (10 mg/mL in 70% ethanol).

8.2 Cycloheximide (10 mg/mL in distilled water).

8.3 Sodium citrate buffer (0.1M, pH 4.80).

8.4 Cellulase enzyme of known activity, FPU/mL.

8.5 -glucosidase enzyme of known activity, pNPGU/mL.

8.6 Solka Floc 200 NF, FCC (microcrystalline cellulose) from Brown Company with ash, moisture, and xylan contents determined (see Ethanol Project Laboratory Analytical Procedures, LAP-001, -002, and -005).

8.7 Eppendorf Safe-Lock 1.5-mL microcentrifuge tubes.

8.8 20-mL glass scintillation vials equipped with plastic-lined caps.

9. ES&H Considerations and Hazards

9.1 Cycloheximide and tetracycline are hazardous and must be handled with appropriate care.

9.2 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

10. Procedure

10.1 Total solids must be determined for all cellulose containing samples to be digested (LAP-001).

Note: all lignocellulosic materials which have undergone some aqueous pretreatment must never undergo any drying whatsoever prior to enzyme digestibility, since irreversible pore collapse can occur in the micro-structure of the biomass leading to decreased enzymatic release of glucose from the cellulose. Additionally, all frozen lignocellulosic materials which are to be subjected to digestibility tests can not have been frozen for more than one month prior to analysis, since, depending on the environment, sublimation could have occurred, leading to possible irreversible collapse of micropores in the biomass.

10.2 Weigh out a biomass sample equal to the equivalent of 0.1 g of cellulose on a 105^{o}C dry weight basis (the cellulose content of the sample is initially determined as glucose by LAP- 002, minus the contribution of any starch present, LAP-016) and add to a 20 mL glass scintillation vial. Also, weigh out 0.1 g of the Solka Floc MVS and add to another vial.

10.3 To each vial, add 5.0 mL 0.1 M, pH 4.8 sodium citrate buffer.

10.4 To each vial, add 40 µL (400 Fg) tetracycline and 30 µL (300 µg) cycloheximide to prevent the growth of organisms during the digestion.

10.5 Calculate the amount of distilled water needed to bring the total volume in each vial to 10.00 mL after addition of the enzymes specified in the following step. Add the appropriate calculated volume of water to each vial. All solutions and the biomass are assumed to have a specific gravity of 1.000 g/mL. Thus, if 0.200 g of biomass is added to the vial, it is assumed to occupy 0.200 mL and 9.733 mL of liquid is to be added.

10.6 Bring the contents of each vial to 50°C by warming in the incubator set at 50° ± 1°C. To each vial is added an appropriate volume of the cellulase enzyme preparation to equal approximately 60 FPU/g cellulose and the appropriate volume of β-glucosidase enzyme to equal 64 *p*NPGU/g cellulose.

> Note: If the rate of enzymatic release of glucose is to be measured, all contents of the vial prior to the addition of the enzyme must be at 50°C. The enzymes are always added last since the reaction is initiated by the addition of enzyme.

10.7 Prepare a reaction blank for the substrate. The substrate blank contains buffer, water, and the identical amount of substrate in 10.00 mL volume.

10.8 Prepare enzyme blanks for cellulase and β-glucosidase with buffer, water, and the identical amount of the enzyme.

10.9 Close the vials tightly and place them in the "Roto-Torque" fixed speed rotator set at an approximate angle of 45°C that has been placed in the VWR incubator set at 50°C. Incubate with gentle rotation (68 RPM) for a period of 72 to 168 hours or until the release of soluble sugars from the sample(s) becomes negligible when measured by YSI, as described in the next step.

10.10 If the progress of the reaction is to be measured, a 0.3-0.5 mL aliquot is removed at each predetermined time interval after the vial contents have been well mixed by shaking. This is accomplished by using a 1.0-mL Eppendorf Pipetman pipet with the tip of the plastic 1.0-mL tip slightly cut off (to allow solids, as well as liquid, to be withdrawn into the orifice). The sample is expelled into a 1.5-mL microcentrifuge tube and centrifuged for 1.5 minutes. The supernatant is subjected to glucose analysis using the YSI glucose analyzer.

11. Calculations

11.1 To calculate the percent digestibility of the cellulose added to the scintillation vial, determine glucose concentration in the centrifuged supernatant by YSI. Subtract the glucose concentrations, if any, from the substrates and enzyme blanks.

11.2 Correct for hydration (multiply the glucose reading by 0.9 to correct for the water molecule added upon hydrolysis of the cellulose polymer) and multiply by 10 mL total volume of assay.

Example: If the glucose analyzer reading (corrected with blanks) is 9.9 mg/mL, then the amount of cellulose digested is:

$$0.0099 \text{ g/mL} \times 10 \text{ mL} \times 0.9 = 0.0891 \text{ g}$$

11.3 Calculate percent digestion:

$$\% \, digestion = \frac{grams \, cellulose \, digested}{grams \, cellulose \, added} \, x \, 100$$

12. Report

12.1 Report the percent cellulose digested in the sample, to two decimal places, on a 105°C dry weight basis. Cite the basis used in the report.

12.2 For replicate analyses of the same sample, report the average, standard deviation, and relative percent difference (RPD).

13. Precision and Bias

13.1 The precision of this protocol has not been defined because it is dependent upon cellulase source and substrate composition. Not only will different preparations of cellulase hydrolyze identical substrates to different extents, but different preparations of pretreated biomass exhibit different amounts of homogeneity.

14. Quality Control

14.1 *Reported significant figures:* Typically results are reported as percentages, calculated to two decimal places, along with the standard deviation and RPD. The assay conditions, specifically digestion time, must be defined when reporting the results.

14.2 *Replicates:* It is recommended the samples be run in duplicate to verify reproducibility.

14.3 *Blank:* Enzyme and substrate blanks are run to correct for glucose contributions other than that produced by cellulose hydrolysis.

14.4 *Relative percent difference criteria:* Not defined; dependent on the substrate being tested. Different preparations of pretreated biomass will exhibit different amounts of homogeneity, which will influence the extent to which they are hydrolyzed.

14.5 *Method verification standard:* Solka Floc 200 NF is digested alongside the samples. Hydrolysis is expected to be in the range of 94.00 - 96.00%.

14.6 *Calibration verification standard:* Not applicable.

14.7 *Sample size:* Dependent upon percent dry weight cellulose composition. Typically between 0.10 and 1.00 grams of sample will be required.

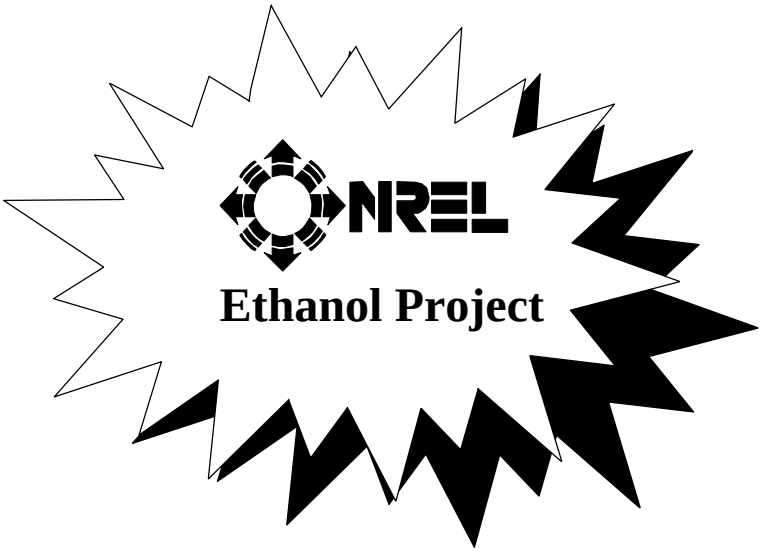
14.8 *Sample storage:* Pretreated samples should be stored moist, or frozen not longer than one month.

14.9 *Standard storage:* Not applicable.

14.10 *Standard preparation:* Not applicable.

14.11 *Definition of a batch*: A batch is defined as the sample replicates and method verification standard hydrolyzed with an identical cellulase preparation and incubated during the same time.

14.12 *Control charts:* Percent hydrolysis of Solka Floc 200 NF will be charted, use of different preparations of cellulase enzyme and total hydrolysis time will be noted.



Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-010

Procedure Title: Standard Method for the Determination of Extractives in Biomass

Author: Tina Ehrman

Date: 4/22/94

ISSUE DATE: 4/22/94 **SUPERSEDES:** n/a

Standard Method for the Determination of Extractives In Biomass

Laboratory Analytical Procedure #010

1. Introduction

1.1 With many types of biomass feedstocks it is necessary that the extractives be removed from the sample prior to analysis to prevent interference with the analytical procedure. Historically, ethanol-benzene has been used to extract waxes, fats, some resins, and portions of wood gums. Subsequent hot water extractions were then used to remove tannins, gums, sugars, starches, and coloring matter. Soxhlet extraction with 95% ethanol has been found to be an effective, non-toxic alternative to extractions employing benzene.

1.2 This procedure has been accepted by ASTM as an ASTM Standard Test Method for the determination of extractives in biomass feedstocks.

2. Scope

2.1 This test method covers the determination of ethanol soluble extractives, expressed as the percentage of the oven-dried biomass, of hard and soft woods, herbaceous materials, agricultural residues, and wastepaper.

2.2 All analyses shall be performed in accordance with the Ethanol Project Quality Assurance Plan (QAP).

3. References

3.1 ASTM D1105-84, "Method for Preparation of Extractive-Free Wood." In *1993 Annual Book of ASTM Standards, Volume 04.09.* Philadelphia, PA: American Society for Testing and Materials.

3.2 Moore, W., and D. Johnson. 1967. *Procedures for the Chemical Analysis of Wood and Wood Products*. Madison, WI: U.S. Forest Products Laboratory, U.S. Department of Agriculture.

3.3 NREL CAT Task Laboratory Analytical Procedure #001, "Determination of Total Solids and Moisture in Biomass."

3.4 NREL Chemical Technologies Research Branch Procedure #001c, "Determination of Extractives Content."

3.5 TAPPI Test Method T204, "Solvent Extractives of Wood and Pulp." *In Tappi Test Methods.* Atlanta, GA: Technical Association of the Pulp and Paper Industry.

4. Significance and Use

4.1 Extractives, as defined by this procedure, are the fraction of a biomass sample soluble in ethanol and that are left as a residue following exhaustive Soxhlet extraction. Extractives include non-structural components of biomass samples which potentially could interfere with the analysis of the biomass sample, and as such must be removed prior to compositional analysis.

4.2 This method gives results comparable to ASTM Method D1105-84.

5. Apparatus

5.1 *Soxhlet extraction apparatus* - A glass Soxhlet extraction apparatus of suitable size (100 mL) for containing the sample and a 250 mL collection flask is required for the conventional Soxhlet procedure. An automated extraction apparatus (Brinkmann Buchi B-810 or equivalent) with circulating oil bath and associated glassware is required for the automated Soxhlet procedure.

5.2 *Alundum extraction thimbles* - Medium porosity (10 - 15 μm pore), sized to fit the Soxhlet extractor.

5.3 *Analytical balance* - Sensitive to 0.1 mg.

5.4 *Rotary evaporator with vacuum and water bath* - Rotary evaporator equipped with a "bump" trap, condenser, receiving vessel, and vacuum source sufficient to pull a vacuum of less than 150 torr.

5.5 *Vacuum oven or drying oven* - Vacuum oven should be controllable to a temperature of 40 ± 1°C and vacuum of between 75 to 100 torr. If drying oven is used in place of the vacuum oven, the drying oven must be able to maintain 45 ± 2EC.

6. Reagents and Materials

6.1 Ethyl alcohol, 95% in water (190 proof), USP grade.

6.2 Boiling chips.

6.3 Glass wool.

6.4 Buchner funnel.

6.5 Desiccator.

7. ES&H Considerations and Hazards

7.1 Ethanol is a flammable reagent.

7.2 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

8. Sampling and Test Specimens

8.1 The test specimen shall consist of approximately 10 grams of milled sample obtained in such a manner as to ensure that it is representative of the entire lot of material being tested.

8.2 If the sample requires milling prior to extraction, pass the sample through a 40 mesh screen (a laboratory scale Wiley mill is recommended for this milling step). Wet samples will require air drying prior to milling.

9. Procedure

9.1 Dry the Soxhlet extraction thimble at 105°C to constant weight. Remove, cool to room temperature in a desiccator, and weigh to the nearest 0.1 mg.

9.2 Carefully add the sample to the extraction thimble. Do not overfill the thimble, leave at least a 1 cm gap between the sample and the top of the thimble. Weigh the filled thimble to the nearest 0.1 mg. Place a plug of glass wool on top of the sample to prevent sample loss during the extraction.

> Note: Samples for total solids determination (following Laboratory Analytical Procedure #001, Determination of Total Solids and Moisture in Biomass) must be weighed out at the same time as the samples for the extractives determination. If this determination is done at a later time, an error in the calculation of the amount of extractives will be introduced, since the moisture content of a biomass sample can change rapidly when exposed to air.

9.3 Place several boiling chips into a clean, dry receiving flask or beaker. Weigh the container, with chips, to the nearest 0.1 mg and record as the tare weight of the container.

9.4 For a conventional Soxhlet extraction (this procedure was reproduced from the Chemical Technologies Research Branch Procedure #001c, Determination of Extractives Content):

9.4.1 Assemble the Soxhlet apparatus using at least 160 mL of 95% ethanol. Insert the thimble and heat at reflux for 24 hours. Periodically check the reflux rate and adjust the heating rate to give four to five solvent exchanges per hour in

the Soxhlet thimble. Approximately 100-120 solvent exchanges are required during the 24 hour period.

9.4.2 When the extraction time is complete, remove the thimble and carefully transfer the sample to a Buchner funnel. Remove any residual solvent by vacuum filtration and wash the sample thoroughly with 95% ethanol, collecting all of the filtrate. Allow the biomass to air dry in the Buchner funnel while it is still attached to the vacuum system.

9.4.3 Combine the filtrate from the previous step and any solvent from the upper section of the Soxhlet apparatus with the solvent in the 250 mL flask. Place the flask on the rotary evaporator and remove the solvent under vacuum. Use a water bath temperature of 45 ± 5°C to heat the flask during evaporation.

9.4.4 After all of the visible solvent is removed by the rotary evaporator, place the flask in a vacuum oven (75-100 torr) at 40 ± 1°C for 24 ± 1 hour. Remove the flask at this time and allow to cool to room temperature in a desiccator. Weigh the flask and record this total weight to the nearest 0.1 mg.

9.5 For an automated Soxhlet extraction:

9.5.1 Turn on the circulating oil bath, and set to 170^{o}C.

9.5.2 Add approximately 100 mL of 95% ethanol to the receiving beaker. Place the thimble containing the sample inside the extractor tube. Finish assembling the automated Soxhlet extractor as directed in the instrument manual.

9.5.3 Begin the extraction, verifying that the reflux rate is ten to twelve solvent exchanges per hour. Reflux for eight hours, giving a total of 80 to 100 solvent exchanges.

> Note: An overnight extraction may be used. Select a circulating oil bath temperature (and, indirectly, a solvent exchange rate) that will produce approximately 100 solvent exchanges during the extraction period. Verify that the apparatus is free of leaks so that there is no loss of solvent during the period of unattended operation.

9.5.4 At the end of the extraction, remove the thimble and transfer to a Buchner funnel. Remove any residual solvent by vacuum filtration and wash the sample thoroughly with 95% ethanol, collecting all of the filtrate. Allow the biomass to air dry in the Buchner funnel while it is still attached to the vacuum system.

9.5.5 Combine the filtrate, the solvent from the extractor tube, and the solvent in the beaker. Evaporate to dryness using a rotary evaporator, as described in the conventional Soxhlet extraction section, or alternatively by using the automated extraction apparatus, as described in the next two steps.

9.5.6 Leaving the beaker in place on the heating block, decrease the temperature of the circulating oil bath to 100°C. Evaporate away the solvent until only about 10 mL remains.

9.5.7 Place the beaker in a drying oven set (45°C) or vacuum oven (75-100 torr and 40°C) for 24 ± 1 hour. Remove the beaker, cool to room temperature in a desiccator, and weigh to the nearest 0.1 mg.

10. Calculations

10.1 Calculate the oven dry weight of the sample, using the average total solids content as determined by the Laboratory Analytical Procedure #001, Determination of Total Solids and Moisture in Biomass.

$$ODW = \frac{(Weight, thimble\ plus\ sample - Weight, thimble)\ x\ \%\ Total\ solids}{100}$$

$$\%\ Extractives = \frac{Weight\ container\ plus\ residue - Tare\ wt.container}{ODW}\ x\ 100$$

10.2 Calculate the amount of extractives in the sample, on a percent dry weight basis.

11. Report

11.1 Report the average percent extractives in the sample on an 105°C dried weight basis, along with the standard deviation and the relative percent difference.

12. Precision and Bias

12.1 Data obtained by replicate testing of a hybrid poplar sample in one laboratory gave a standard deviation in extractive content of 0.15% and a CV% of 7.6%. Replicate testing of a National Institute of Standards and Technology (NIST) #8494 wheat straw gave a standard deviation of 0.20% and a CV% of 1.6% and NIST #8493 Pinus radiata gave a standard deviation of 0.20% and a CV% of 8.0%.

12.2 Prolonged heating of the extractive residue may bias the reported results low because of evaporation of semivolatile constituents. Insufficient heating or using inadequate vacuum can bias the results high because of incomplete removal of the ethanol solvent.

13 Quality Control

13.1 *Reported significant figures:* All results shall be reported as a percentage with two decimal places.

13.2 *Replicates:* All samples and method verification standards shall be analyzed in duplicate.

13.3 *Blank:* It is recommended that a solvent blank be run with every batch of samples.

13.4 *Relative percent difference criteria:* The %RPD must be less than 10%. If the %RPD is too large, the sample will be rerun.

13.5 *Method verification standard:* It is recommended that a method verification standard be run with every batch. This standard is a material of known extractives content that is run in parallel with the samples to track the reproducibility of the analysis.

13.6 *Calibration verification standard:* Not applicable.

13.7 *Sample size:* The sample, added to an extraction thimble, shall be a minimum of three grams or the results will be flagged as having compromised precision.

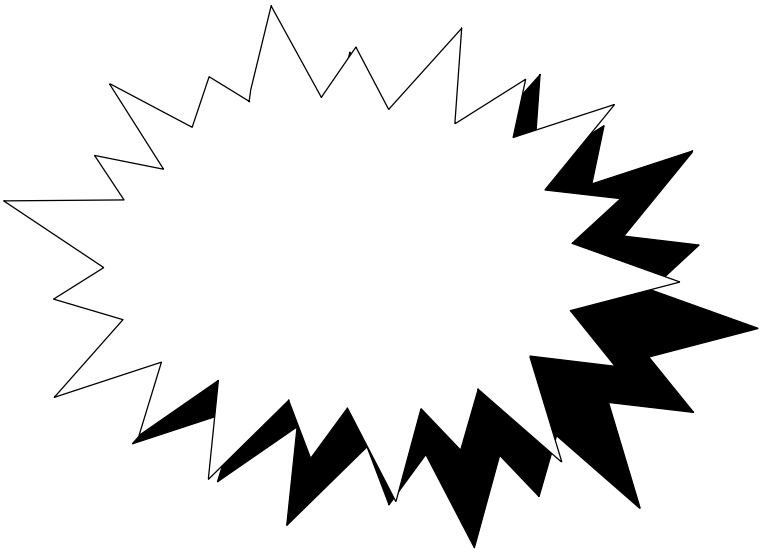
13.8 *Sample storage:* Store the extracted sample in the refrigerator until needed for further analysis.

13.9 *Standard storage:* Not applicable.

13.10 *Standard preparation:* Not applicable.

13.11 *Definition of a batch*: Any number of samples which are analyzed together and recorded together. The maximum size of a batch is limited by the equipment constraints. A batch cannot be larger than what is practical with the available equipment.

13.12 *Control charts:* All method verification standards shall be control charted.



Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-011

Procedure Title: Determination of Ethanol Concentration in Biomass to Ethanol Fermentation Supernatants by Gas Chromatography

Author: David W. Templeton

Date: 5/5/94

ISSUE DATE: 5/5/94 **SUPERSEDES:** n/a

Determination of Ethanol Concentration in Biomass to Ethanol Fermentation Supernatants by Gas Chromatography

Laboratory Analytical Procedure #011

1 Introduction

1.1 Biomass is thought to be a good substrate for bioconversion to ethanol. This ethanol could be used as a more environmentally benign transportation fuel. Various processes have been developed to convert biomass to ethanol. The efficiency of a process can be determined by measuring the yield of ethanol. Gas Chromatography (GC) is a fast and accurate means of determining volatile components such as ethanol.

2 Scope

2.1 This procedure details a method to prepare biomass to ethanol fermentation supernatants for analysis and quantify their ethanol concentration. This packed column GC method utilizes isopropanol as an internal standard to determine concentration, in g/L, of ethanol in the fermentation supernatant. This method is capable of analyzing fermentation supernatant ethanol concentrations ranging from 1 to 50 g/L.

2.2 This procedure describes an associated QA/QC program to demonstrate that the results comply with the Ethanol Project Quality Assurance Plan. Adherence to the Quality Assurance Plan is assumed for all work associated with this procedure.

3 Terminology

3.1 *Biomass to ethanol fermentation*-A process accomplished by enzymatically breaking down biomass structural polymers to monomeric sugars, and utilizing yeast to ferment these sugars to ethanol. Simultaneous saccharification and fermentation (SSF) is one method used to convert biomass to ethanol.

3.2 *Fermentation supernatant*-The liquified portion of a biomass to ethanol fermentation, including cells and biomass debris. For the purposes of the procedure, this refers to slurry samples prior to preparation for analysis.

3.3 *Analytical sample*-A fermentation supernatant that has been filtered and diluted with internal standard for GC analysis.

4. Significance and Use

4.1 Ethanol is the desired product of the biomass to ethanol fermentation process. Accurate quantitation of ethanol concentration is crucial to the design, assessment, and improvement of the process.

5. Interferences

5.1 Biomass to ethanol fermentation supernatants contain high concentrations of organic and inorganic residue. Much of this residue is not volatile and remains in the injection port leading to fouling and activation. An activated injection port can cause the catalytic breakdown of the analytes of interest, before they are detected, adversely affecting the quantitation.

5.2 The biomass to ethanol fermentation process can produce a wide variety of volatile compounds some of which may co-elute with ethanol or isopropanol. This can adversely affect the quantitation.

6. Apparatus

6.1 *Gas Chromatograph (HP 5890 or equivalent)*-Utilizing a flame ionization detector (FID), removable, deactivated glass, injection port liner, associated regulators, gas lines, and septa.

6.2 *Packed GC Column*-6'x1/8" stainless steel Porapak Q (HP 19001A-Q00 or equivalent).

6.3 *Autoinjector (HP 7673 or equivalent)*-Recommended.

6.4 *Data handling system (HP Chemstation or equivalent)*-Typically computer controlled, but may be an integrator or chart recorder.

6.5 *Centrifuge*.

6.6 *Pan balance*-Readable to 0.01g.

7. Reagents and Materials

7.1 *Isopropanol*-Reagent grade.

7.2 *Ethanol*-200 proof.

7.3 *Ethanol*-Separate source, either a 200 proof ethanol standard from a different manufacturer than (7.2), or a commercially prepared solution of known concentration.

> *Note:* Store isopropanol and ethanol reagents appropriately, protect from evaporation and moisture.

7.4 *UHP nitrogen*-GC carrier gas.

7.5 *UHP hydrogen*-FID fuel.

7.6 *Zero air*-FID oxidant.

7.7 *Water*-Reagent grade.

7.8 *Repeat pipette(s)*-Capable of delivering appropriate volumes.

7.9 *Autosampler vials (2 ml) with associated caps.*

7.10 *Crimper*-If necessary.

7.11 *Capped centrifuge tubes.*

7.12 *0.45 mm filter*-Either in line syringe filter unit or insert for centrifuge tube.

7.13 *Assorted class A volumetric pipette and flasks*.

7.14 *Paper towel(s).*

8. ES&H Considerations and Hazards

8.1 Utilize caution when handling compressed gasses (especially compressed hydrogen).

8.2 Ethanol and isopropanol are flammable reagents.

8.3 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

9. Sampling, Test Specimens and Test Units

9.1 Sample the fermentation so as to collect a representative slurry sample.

9.2 If collecting several samples, chill collected supernatants on ice while continuing to collect more.

9.3 Fermentation slurries are to be centrifuged within one hour of sampling. This can be accomplished by spinning at 6000 RPM for five minutes.

9.4 Filter the liquid portion of the sample through a 0.45 μm filter.

9.5 Dilute each filtered fermentation supernatant into a labeled autosampler vial. For typical fermentation supernatants, a tenfold dilution brings the ethanol concentration in the analytical sample within the linear range of the calibration curve. In this case, 100 μL of filtered fermentation supernatant is added, using a suitable repeat pipette, to 900 μL internal standard spiking solution. If the concentration of the analytical sample falls outside of the linear range, re-dilute the sample accordingly.

9.6 For every 25 analytical samples, prepare a sample in duplicate. Duplicates are used to analyze the method precision.

9.7 For every 25 analytical samples, prepare a method verification standard by pipetting 100 μL intermediate calibration verification solution into 900 μL internal standard spiking solution. This method verification standard is compared to the concentration verification standard to verify confidence in the pipetting accuracy and technique.

> *Note:* Regularly check the accuracy of repeat pipettes; they can be the source of large errors.

9.8 Unless the same internal standard spiking solution is used for both analytical standards and samples, prepare an internal standard check sample one for every 25 samples. Prepare by pipetting 100 μL intermediate calibration verification solution into 900 μL of the internal standard spiking solution used for analytical samples. The internal standard check sample is used to compare the different internal standard spiking solutions.

9.9 Cap the autosampler vial snugly.

9.10 Refrigerate all samples. Analyze samples as soon as practicable. Do not store analytical samples, prior to analysis, for more then one month.

10. Preparation of Apparatus

10.1 Clean the injection syringe before starting the analysis. Biomass to ethanol fermentation supernatants tend to leave a sticky film on the plunger which affects the precise operation of the syringe.

10.1.1 Remove the plunger from the syringe and wipe down using a paper towel soaked in reagent grade water.

10.1.2 Place a few drops of reagent grade water on top of syringe and force several volumes through with the plunger.

10.2 If possible, utilize any autosampler's self cleaning ability to extensively clean the syringe between injections.

10.3 Change the deactivated glass injection port liner frequently. Typically the isopropanol internal standard area counts begin to fall soon after running fermentation samples. After the first twenty or so analytical sample injections, these values tend to stabilize at a lower sensitivity. It is in this stabilized region that analysis takes place. After approximately 100 additional injections the isopropanol internal standard area counts become unstable and analytical precision is compromised. The injection port liner must be changed at this time.

10.4 Change the GC septum after every 50 injections or so.

11. Calibration and Standardization

11.1 Prepare appropriate concentration internal standard spiking solution, using reagent grade isopropanol. The internal standard spiking solution must be added in the same proportion to every standard or sample analyzed by this method. This procedure specifies nine parts of a 1 g/L internal standard spiking solution be added to one part sample or standard. Therefore, the internal standard concentration is 0.9 g/L universally throughout this procedure.

11.2 Prepare 3 to 6 ethanol analytical standards, using 200 proof ethanol, ranging from 0.1 to 5.0 g/L and all containing 0.9 g/L isopropanol.

11.3 Prepare 1-3 intermediate calibration verification solutions from the separate ethanol source. Dilute 1:10 with internal standard spiking solution, using a volumetric pipette and volumetric flask. When diluted in this manner, the intermediate calibration verification solution becomes a calibration verification standard. The concentration of the calibration verification standard(s) are to be within the range of the analytical standards, but not equal to any of them.

11.3.1 Refrigerate analytical and calibration verification standards in sealed autosampler vials for storage. Properly sealed, the analytical and calibration standards are good for three months.

11.4 Prepare several solvent washes by dispensing reagent grade water into an autosampler vial and seal.

12. Conditions

12.1	Oven temperature:	155^{o}C (isothermal).
12.2	Inlet temperature:	175^{o}C.
12.3	Detector temperature:	250^{o}C.
12.4	Run time:	5.5 minutes.
12.5	Ethanol retention time:	About 2.3 minutes.
12.6	Isopropanol retention time:	About 4.1 minutes.
12.7	Carrier gas flow rate:	30 ml/min.
12.8	FID flow rates:	Per manufacturer's recommendations.
12.9	Injection volume:	1 µl.

13. Procedure

13.1 Warm all standards and samples to room temperature and lightly mix the contents of all the vials.

13.2 Set up analytical run as follows:

13.2.1 Solvent wash.

13.2.2 Analytical standards from low concentration to high.

13.2.3 Calibration verification standard.

13.2.4 Solvent wash.

13.2.5 A batch of samples in randomized order. A batch, typically, is a set of samples from one time point, including associated method verification standards, replicates, and internal standard check standards. A batch usually does not exceed 20 samples.

13.2.6 Calibration verification standard.

13.2.7 Solvent wash.

13.2.8 Repeat 13.2.5-13.2.7, if necessary.

13.3 Recalibrate the standard curve after every 6 hours of analysis.

13.4 Do not reuse analytical or calibration verification standards.

14. Calculations

14.1 This method utilizes an internal standard which corrects for variations in the injection volume.

14.2 Typically analytical software is utilized to perform concentration calculations. The chromatograms are integrated based upon peak area. The calibration curve is generated by a linear regression of the analytical standards, and ignores the origin as a calibration point. Confirm the linearity of the calibration curve; the coefficient of variation of the linear regression must be greater than 0.99.

14.3 If analytical software is not utilized, the calculation may be done manually. From the standards data, create a calibration curve of amount ratio, plotted on the abscissa, versus response ratio, plotted on the ordinate. The amount ratio is the ethanol concentration divided by the isopropanol internal standard concentration. The response ratio is the ethanol area divided by the isopropanol area.

Calculate a linear regression through the standard points, excluding 0,0 as a data point. The equation of the resulting line should take the form of :
$response\ ratio = slope\,(amount\ ratio) + (y intercept)$ For the analytical samples, the response ratio is determined and the internal standard concentration is known. Use the equation, above, to solve for the ethanol concentration in the samples.

14.4 The calculated fermentation supernatant concentration needs to account for any dilution that occurred during the preparation of the analytical sample. In this case, multiply the ethanol concentration of the analytical sample by ten.

15. Report

15.1 Report all results to the nearest 0.1 g/L.

16. Precision and Bias and Quality Control

16.1 Analysis in one laboratory of a calibration verification standard at the lower end of the analytical range showed a recovery of 100.32% with a coefficient of variation of 3.60%.

16.2 Analysis in one laboratory of a calibration verification standard at the higher end of the analytical range showed a recovery of 97.93% with a coefficient of variation of 4.67%.

17 Quality Control

17.1 *Reported significant figures:* Report all results to the nearest 0.1 g/L according to section 15.1.

17.2 *Replicates:* Prepare one replicate for every 25 samples prepared according to section 9.6.

17.3 *Blank*: Prepared according to section 11.4. The solvent washes are used check for any carryover problems. There should be no ethanol or isopropanol peaks in the solvent wash runs.

17.4 *Relative percent difference (rpd) criteria*: Calculate the rpd for each duplicate set. Limits for rpd have not been determined precisely, but a rpd greater than 15% is cause for reanalysis.

17.5 *Method verification standard*: Prepared one for every 25 samples prepared according to section 9.7.

17.6 *Calibration verification standard*: Prepared according to section 11.3.

17.7 *Sample size:* 100 µL of filtered fermentation supernatant prepared according to section 9.5.

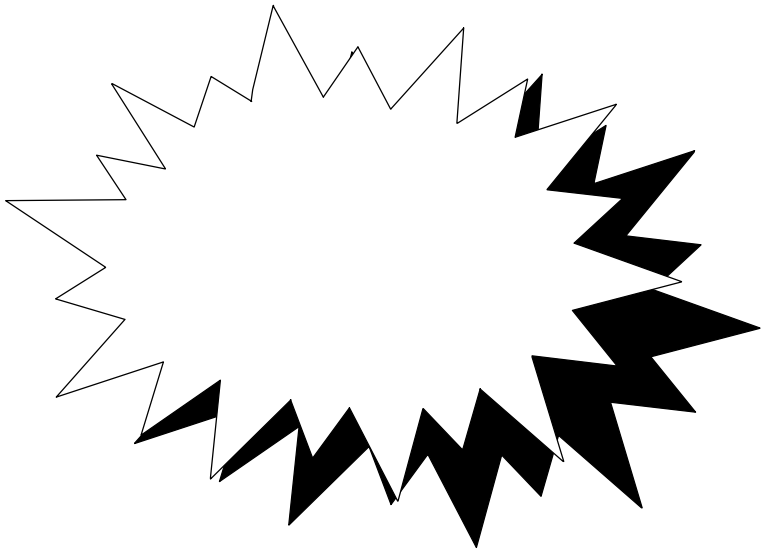
17.8 *Sample storage*: Refrigerated in autosampler vials for no more than a month according to section 9.10.

17.9 *Standard preparation*: Per section 11.

17.10 *Batch size*: Defined in section 13.2.5.

17.11 *Control Charts*: Calculate the percent recovery for each calibration verification standard run and control chart these values. Control chart the slope of the analytical curve.

17.12 *Matrix effects:* It is assumed that the matrix (fermentation media) for these sample will not vary greatly. If the matrix is considerably changed, it must be shown that the new matrix does not affect the accuracy of this method.



Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-012

Procedure Title: Standard Test Method for Moisture, Total Solids, and Total Dissolved Solids in Biomass Slurry and Liquid Process Samples

Author: Tina Ehrman

Date: 6/13/94

ISSUE DATE: 7/5/94 **SUPERSEDES:** n/a

Standard Test Method for Moisture, Total Solids, and Total Dissolved Solids in Biomass Slurry and Liquid Process Samples

Laboratory Analytical Procedure #012

1. Introduction

1.1 A critical component of evaluating processes involved in the conversion of biomass is the accurate compositional analysis of samples from those process streams. Although these process samples can be analyzed as a whole sample (slurry), often they are separated into solid and liquid fractions and analyzed separately. To be meaningful, the results of chemical analyses of biomass are typically reported on a dry weight basis. The following procedure describes the method used to determine the total solids (or moisture) content of a slurry or the liquid fraction of a biomass process sample. This procedure can also be used to determine the total dissolved solids content of such samples.

2. Scope

2.1 This test method covers the determination of total solids (or moisture) in slurries or the liquid fraction of samples generated during the pretreatment, fractionation, and fermentation of biomass.

3. References

3.1 Moore, W.E., and D.B. Johnson. 1967. *Procedures for the Chemical Analysis of Wood and Wood Products*. Madison, WI: USDA Forest Products Laboratory, U.S. Department of Agriculture.

3.2 Technical Association of the Pulp and Paper Industry (TAPPI) Standard Method T OS-63.

3.3 NREL Chemical Analysis and Testing Task Laboratory Analytical Procedure #001, "Standard Test Method for the Determination of Total Solids in Biomass".

4. Terminology

4.1 The total solids content of a sample is the amount of material left as a residue upon drying at 105^{o}C to constant weight.

4.2 The total dissolved solids content applies to liquid or slurry samples and is defined in

this procedure as the amount of residue from the filtrate of a 0.2 μm filtered sample that has been dried at 105°C to constant weight.

5. Significance and Use

5.1 The total solids content is a measure of the amount of solids suspended or dissolved in a process liquid or slurry. Conversely the moisture content is a measure of the amount of water (and other components volatilized at 105°C) present in such samples.

5.2 The results of chemical analyses of processed biomass samples are typically reported on a dry weight basis. The total solids content of a sample is used to convert the analytical results obtained on another basis to that of a dry weight basis.

6. Apparatus

6.1 Automatic infrared moisture analyzer (such as Denver Instrument Company IR-100 or equivalent) or a convection oven, with temperature control of 105 ± 2°C.

6.2 Analytical balance, sensitive to 0.1 mg.

6.3 Desiccator.

7. Reagents and Materials

7.1 Syringe filter, 0.8 μm prefilter over 0.2 μm final filter (such as Gelman Acrodisc μF or equivalent).

7.2 Aluminum foil weighing dishes.

7.3 Quartz pads for weighing dishes.

7.4 Transfer pipettes.

8. ES&H Considerations and Hazards

8.1 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

9. Sampling, Test Specimens and Test Units

9.1 Test specimens suitable for analysis by this procedure are slurries and the liquid fraction of samples generated during the pretreatment, fractionation, or fermentation of biomass. If the total solids (or moisture) contents of the solid fraction of these process samples are to be determined, the Laboratory Analytical Procedure #001, "Standard Method for the Determination of Total Solids in Biomass", must be used instead.

9.2 The test specimen shall consist of approximately 3 to 10 g of sample obtained in such a manner as to ensure that it is representative of the entire lot of material being tested. Thorough mixing of slurries and liquid fractions with precipitates is of particular importance.

10. Convection Oven Procedure

10.1 Accurately weigh a predried aluminum foil weighing dish to the nearest 0.1 mg and then tare the balance.

10.2 For a total solids or moisture determination, thoroughly mix the sample and then weigh out 3 to 10 g, to the nearest 0.1 mg, into the tared weighing dish. For total dissolved solids, the sample added to the tared weighing dish should first be passed through a 0.8/0.2 μm syringe filter.

10.3 Place the sample into a convection oven at 105 ± 2°C and dry to constant weight (±0.1% change in the amount of moisture present upon one hour of reheating). Typically overnight drying is required for very wet samples.

10.4 Remove the sample from the oven and place in a desiccator; cool to room temperature.

10.5 Weigh the dish containing the oven-dried sample to the nearest 0.1 mg.

11. Infrared Moisture Analyzer Procedure

11.1 Program the automatic moisture analyzer for an analysis temperature of 105°C and for a pre-determined end of analysis criteria of a rate of weight change that does not exceed 0.05% in one minute.

11.2 Verify that the instrument has reached the analysis temperature of 105°C and then place an aluminum foil weighing dish with quartz pad on the balance pan. Wait five minutes to ensure that the dish and pad are completely dry and then tare the balance.

11.3 For a total solids or moisture determination, quickly transfer 3 to 10 g of the thoroughly mixed sample to the quartz pad in the weighing dish. For total dissolved solids, the sample added to the quartz pad should first be passed through a 0.8/0.2 μm syringe filter.

11.4 As soon as the instrument balance produces a stable weight, proceed with the analysis.

11.5 Once the sample has been dried to constant weight, as determined by the programmed analysis parameters, the analysis will be automatically terminated by the instrument.

12. Calculations

12.1 Calculate the percent total solids or the percent moisture on a 105°C dry weight basis as follows (the automated moisture analyzer will provide the selected calculated value as part of the instrument printout):

$$\%\ Total\ solids = \frac{weight\ dried\ sample\ plus\ dish - weight\ dish}{weight\ sample\ as\ received} \times 100$$

$$\%\ Moisture = [1 - \frac{(weight\ dried\ sample\ plus\ dish - weight\ dish)}{weight\ sample\ as\ received}] \times 100$$

12.2 Calculate the percent total dissolved solids on a 105°C dry weight basis as follows (the automated moisture analyzer will provide the selected calculated value as part of the instrument printout):

$$\%\ Total\ dissolved\ solids = \frac{weight\ dried\ sample\ plus\ dish - weight\ dish}{weight\ sample\ after\ 0.8/0.2\ um\ \ filtration} \times 100$$

13. Report

13.1 Report the result as a percentage with two decimal places.

13.2 For replicate analyses of the same sample, report the average, standard deviation, and relative percentage difference.

14. Precision and Bias

14.1 The precision of this analysis is determined by evaluation of %RPD data from samples of many different types and assumes a 95% confidence interval. Based on this evaluation, the precision of the infrared moisture analyzer procedure is 3% using a Denver Instrument IR-100. The precision for the convection oven procedure is 1%.

14.2 An inherent error in any oven drying procedure is that volatile substances other than water are removed from the sample during drying.

15. Quality Control

15.1 *Reported significant figures:* All data is reported with two decimal places.

15.2 *Replicates:* All samples are run in duplicate.

15.3 *Relative percent difference criteria:* For the infrared drying method the maximum %RPD for duplicate analysis of a liquid samples is 9%. For the oven method the maximum %RPD is 3%. If the %RPD is exceeded, the sample should be rerun.

15.4 *Blank:* This gravimetric analysis utilizes a balance blank with every batch of samples, consisting of a weighing dish passed through all steps of the procedure.

15.5 *Method verification standard:* A method verification standard should be run with every batch of samples. A solution containing 2% (w/v) sodium chloride can be prepared and used for this purpose, provided it is stored in a tightly sealed container. Process 5.0 mL of this solution in the same manner as a sample.

15.6 *Calibration verification standard:* Not applicable.

15.7 *Definition of a batch:* Any number of samples which are analyzed together and recorded together. Samples within a batch must be of the same matrix. The maximum size of a batch would be limited by the equipment constraints. A batch cannot be larger than what is practical with the equipment.

15.8 *Sample size:* 10 mL sample minimum, 15 mL recommended.

15.9 *Sample storage:* Samples should be refrigerated.

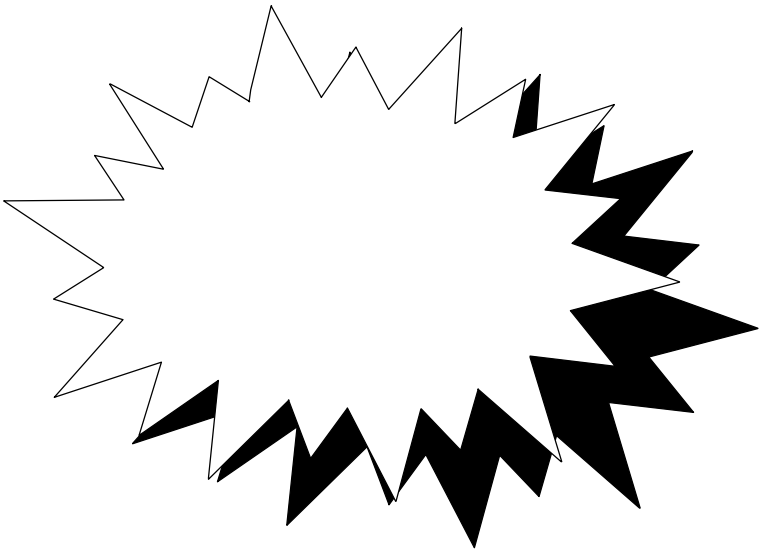
15.10 *Standard storage:* Not applicable.

15.11 *Standard preparation:* Not applicable.

15.12 *Control charts:* The results of the method verification standard are to be control charted.

16. Keywords

16.1 Moisture, total solids, biomass.



Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-013

Procedure Title: HPLC Analysis of Liquid Fractions of Process Samples for Monomeric Sugars and Cellobiose

Author: Raymond Ruiz and Tina Ehrman

Date: 6/28/96

ISSUE DATE: 8/26/96 **SUPERSEDES:** 11/01/94

HPLC Analysis of Liquid Fractions of Process Samples for Monomeric Sugars and Cellobiose

Laboratory Analytical Procedure #013

1. Introduction

1.1 Carbohydrates make up a major portion of biomass samples. These carbohydrates are polysaccharides constructed primarily of glucose, xylose, arabinose, galactose, and mannose monomeric subunits. During the processing of the biomass, such as dilute acid pretreatment, a portion of these polysaccharides are hydrolyzed and soluble sugars released into the liquid stream. Fermentation samples, whether they are time point samples or end point residues, will also contain soluble sugars. The soluble sugars in the liquid fraction of process samples can be quantified by HPLC with refractive index detection.

2. Scope

2.1 This procedure is used to determine the soluble monosaccharide content of the liquid fractions of biomass to ethanol process streams, including pretreatment liquors, liquid fermentation time point samples, and the liquid fraction of fermentation residues. The soluble sugar content indicates the amount of fermentable sugars available for conversion to ethanol at specific process steps.

2.2 All analyses shall be performed according to the guidelines established in the Ethanol Project Quality Assurance Plan (QAP).

3. References

3.1 Ehrman, C.I., and M.E. Himmel. 1994. "Simultaneous Saccharification and Fermentation of Pretreated Biomass: Improving Mass Balance Closure." *Biotechnology Techniques*, 8(2):99-104.

3.2 Moore, W., and D. Johnson. 1967. Procedures for the Chemical Analysis of Wood and Wood Products. Madison, WI: U.S. Forest Products Laboratory, U.S. Department of Agriculture.

4. Significance and Use

4.1 The concentrations of monomeric sugars and cellobiose are used in conjunction with other assays to determine the total composition of process stream samples.

5. Apparatus

5.1 Analytical balance, accurate to 0.1 mg.

5.2 pH meter, readable to 0.01 pH unit.

5.3 HPLC system equipped with refractive index detector.

5.4 Biorad Aminex HPX-87C and/or HPX-87P columns (or analytical HPLC columns shown to give equivalent separations as the Biorad columns). equipped with the appropriate guard columns.

> Note: Deashing guard column cartridges from BioRad, of the ionic form H^+/CO_3^-, are recommended when using an HPX-87P column. These cartridges have been found to be effective in eliminating baseline ramping.

6. Reagents and Materials

6.1 High purity sugars for standards - cellobiose, glucose, xylose, arabinose, galactose, and mannose.

6.2 Second set of the high purity sugars listed above, from a different source (manufacturer or lot) for preparation of calibration verification standards (CVS).

6.3 Calcium carbonate, ACS reagent grade.

6.4 Water, HPLC grade or better, 0.2 μm filtered.

6.5 pH paper (range 2-9).

6.6 Disposable syringes, 3 mL, fitted with 0.2 μm syringe filters.

6.7 Autosampler vials with crimp top seals to fit.

6.8 Volumetric pipets, class A, of appropriate sizes.

6.9 Volumetric flasks, class A, of appropriate sizes.

6.10 Adjustable pipettors, covering ranges of 10 to 1000 µL.

7. ES&H Considerations and Hazards

7.1 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

8. Calibration and Standardization

8.1 This analysis uses a multipoint calibration as described in the procedure.

9. Procedure

9.1 Thoroughly mix the sample and then measure and record the pH of a small aliquot to the nearest 0.01 pH unit. It will be necessary to adjust the pH of the sample if this reading falls outside of the operating pH range (5-9) of the HPLC column to be used.

9.2 Dilute the samples as needed, so the concentration of each sugar falls within the validated range of the analytical method. Prepare each dilution in duplicate.

> Note: It may be useful to determine initial glucose concentrations of the samples using an alternative technique, such as YSI glucose analyzer, in order to predict whether or not the sugars in the sample will fall within the linear range of the analysis. In samples such as pretreatment liquors, xylose typically is present at high concentrations, often three to five times the level of glucose. In other samples, such as the liquid fractions of fermentation samples, the levels of all the soluble sugars can be relatively low, and the samples therefore will not require dilution.

9.3 Prepare method verification standards (MVS) by selecting representative samples to be used to determine spike recoveries. Spike an accurately measured volume of each selected sample with a known amount of the components of interest, such that the final concentrations still fall within the linear range of the analysis. Process these spiked samples along with the rest of the samples.

9.4 If the pH of the sample is less than 5, neutralize duplicate 10 mL aliquots with calcium carbonate to a pH between 5 and 6. Avoid neutralizing to a pH greater than 6 by monitoring with a pH strip. Add the calcium carbonate slowly after about pH 4 and swirl frequently.

> Note: Samples of pH greater than 9, such as alkaline pretreatment liquors, should not be analyzed using the HPX-87C or HPX-87P columns unless the sample pH can be brought into the operating range of the column, pH 5 to 9.

9.5 Pass the appropriately diluted and/or neutralized samples through 0.2 µm syringe filters into autosampler vials in preparation for HPLC analysis. Seal and label the vials. Reserve a portion of the undiluted sample in case repeat analyses are required.

Store the reserved samples in the refrigerator.

9.6 Prepare a series of sugar calibration standards in HPLC grade water at concentrations appropriate for creating a calibration curve for each sugar of interest. A suggested scheme for the HPX-87C column is to prepare a set of multi-component standards containing glucose, xylose, and arabinose in the range of 0.2 - 12.0 mg/mL. For the HPX-87P column, cellobiose, galactose, and mannose should be included as additional components in the standards. Extending the range of the calibration curves beyond 12.0 mg/mL will require validation.

9.7 Prepare an independent calibration verification standard (CVS) for each set of calibration standards, using sugars obtained from a source other than that used in preparing the calibration standards. The CVS must contain precisely known amounts of each sugar contained in the calibration standards, at a concentration that falls in the middle of the validated range of the calibration curve. The CVS is to be analyzed after each calibration curve and at regular intervals in the HPLC sequence, bracketing groups of samples. The CVS is used to verify the quality of the calibration curve(s) throughout the HPLC run.

9.8 Analyze the calibration standards, the calibration verification standards, the samples, and the method verification (spiked) samples by HPLC using a Biorad Aminex HPX-87C or HPX-87P column for glucose, xylose, and arabinose. If cellobiose, mannose, and galactose are also to be determined, only the Biorad Aminex HPX-87P column should be used. For many analyses, it is useful to run the same samples on both columns and compare the results. The following instrumental conditions are used for both the HPX-87C and the HPX-87P columns:

Sample volume: 50 μL.
Eluant: 0.2 μm filtered and degassed HPLC grade water.
Flow rate: 0.6 mL/min.
Column temperature: 85^{o}C.
Detector: refractive index.
Run time: 20 minutes data collection plus a 15 minute post-run.

10. Calculations

10.1 Create a calibration curve for each sugar to be quantified using linear regression. From these curves, determine the concentration in mg/mL of the sugars present in each sample analyzed by HPLC, corrected for dilution.

10.2 Calculate and record the percent recoveries, %CVS, for each sugar contained in the calibration verification standards analyzed by HPLC.

$$\%\ CVS\ recovered = \frac{conc.\,detected\ by\ HPLC, mg/ml}{known\ conc.\,of\ \ CV\ before\ HPLC\ analysis, mg/ml} \ x\ 100$$

10.3 Calculate and record the percent spike recoveries (% recovery MVS) for each sugar used to prepare the method verification standards analyzed by HPLC.

10.3.1 Correct the initial sample concentration for the dilution resulting from the addition of a known volume of spike solution.

$$C_{corrected} = \frac{V_{sample}}{V_{final}} \ x\ C_{sample}$$

Where: V_{sample} = volume of sample prior to spiking, in mL.
V_{final} = final volume of solution (spike plus sample), in mL.
C_{sample} = initial concentration of sample prior to spiking in mg/mL, as determined by HPLC.
$C_{corrected}$ = concentration of sample after being corrected for dilution, in mg/mL.

10.3.2 Calculate the percent recovery of the spike.

$$\%\ Recovery\ MVS = \frac{C_{actual} - C_{corrected}}{C_{spike}} \ x\ 100$$

Where: C_{actual} = actual concentration of spiked sample, as determined by HPLC, in mg/mL.
$C_{corrected}$ = concentration of sample after correcting for dilution, in mg/mL, as calculated above.
C_{spike} = known concentration of spike solution added to sample prior to analysis, in mg/mL.

11. Precision and Bias

11.1 In the determine the cellobiose and monomeric sugar contents of process samples, the neutralized samples are routinely analyzed using the HPX-87P column. When these samples are known not to contain galactose and mannose, the HPX-87C column may be used instead. Based on a root mean square evaluation of duplicate data, there is a 95% certainty that the "true value" will be within the range of the average plus or minus:

- glucose 3.37% (HPX-87C) and 3.12% (HPX-87P),
- xylose 1.92% (HPX-87C) and 5.02% (HPX-87P).

Analytes at or near the detection limit could have significantly higher precision errors.

11.2 Samples containing significant amounts of protein, oligosaccharides, or other compounds which elute early on the HPX-87C and HPX-87P columns will exhibit baseline disturbances which may interfere with the quantification of the analytes.

12. Quality Control

12.1 *Reported significant figures:* Report all results in mg/mL with two decimal places. The standard deviation and relative percent difference are also to be reported.

12.2 *Replicates:* All samples are to be run in duplicate. For replicate analyses of the same sample, report the average, standard deviation, and relative percent difference (RPD).

12.3 *Relative percent difference criteria:* The maximum RPD for duplicate samples is as follows: glucose, 5.8%, and xylose, 8.1%. If the stated RPD is exceeded, the sample should be rerun. However, analytes at or near the detection limit could have significantly higher RPDs.

12.4 *Blank:* The only requirement is an instrumental blank, consisting of the HPLC grade water analyzed by HPLC in the same manner as the samples.

12.5 *Method verification standard:* This method will utilize a matrix spike as the method verification standard, as indicated in the procedure.

12.6 *Calibration verification standard:* Calibration verification standards shall be independently prepared and analyzed as described in the procedure.

12.7 *Definition of a batch:* Any number of samples which are analyzed together and recorded together. Samples within a batch must be of the same matrix. The maximum size of a batch will be limited by the equipment constraints. A batch cannot be larger than what is practical with the equipment.

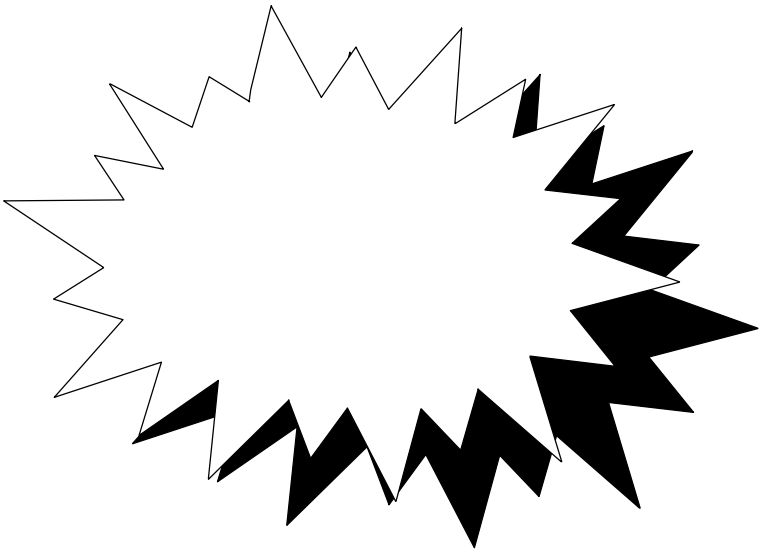
12.8 *Sample size:* 30 mL minimum.

12.9 *Sample storage:* Samples should be refrigerated.

12.10 *Standard storage:* Standards should stored frozen. Upon thawing, the standards should be vortexed p and then shaken after thawing and again prior to use.

12.11 *Standard preparation:* Standards are prepared according to section 8.6 of this procedure.

12.12 *Control charts:* All spike recoveries and calibration verification standards are control charted.



Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-014

Procedure Title: Dilute Acid Hydrolysis Procedure for Determination of Total Sugars in the Liquid Fraction of Process Samples

Author: Raymond Ruiz and Tina Ehrman

Date: 3/6/96

ISSUE DATE: 8/12/96 **SUPERSEDES:** 11/01/94

Dilute Acid Hydrolysis Procedure for Determination of Total Sugars in the Liquid Fraction of Process Samples

Laboratory Analytical Procedure #014

1. Introduction

1.1 Carbohydrates make up a major portion of biomass samples. These carbohydrates are polysaccharides constructed primarily of glucose, xylose, arabinose, galactose, and mannose monomeric subunits. During dilute acid pretreatment of biomass, a portion of these polysaccharides are hydrolyzed and soluble sugars released into the liquid stream. These sugars, if present in oligomeric form, cannot easily be quantified without further processing into their monomeric units.

2. Scope

2.1 This procedure is used to determine the total sugar content, including both monosaccharides plus oligosaccharides, of the liquid fractions of biomass to ethanol process streams, including pretreatment liquors, liquid fermentation time point samples, and the liquid fraction of fermentation residues.

2.2 All analyses shall be performed according to the guidelines established in the Ethanol Project Quality Assurance Plan (QAP).

3. References

3.1 Ehrman, C.I., and M.E. Himmel. 1994. "Simultaneous Saccharification and Fermentation of Pretreated Biomass: Improving Mass Balance Closure." *Biotechnology Techniques*, 8(2):99-104.

3.2 Moore, W., and D. Johnson. 1967. Procedures for the Chemical Analysis of Wood and Wood Products. Madison, WI: U.S. Forest Products Laboratory, U.S. Department of Agriculture.

4. Significance and Use

4.1 The total sugar content is used in conjunction with other assays to determine the total composition of process stream samples.

4.2 The difference between the total sugar content determined by this procedure and the monosaccharide content, determined by LAP #013, can be used to assess the competence of specific process steps and is an indicator of the amount of non-fermentable sugars present at that specific phase of the process.

5. Apparatus

5.1 Analytical balance, accurate to 0.1 mg.

5.2 pH meter, readable to 0.01 pH unit.

5.3 Pan balance, accurate to 0.01 g.

5.4 Autoclave, set to $121^{o} \pm 3^{o}C$.

5.5 HPLC system equipped with refractive index detector.

5.6 Biorad Aminex HPX-87C with corresponding guard column and/or HPX-87P column with deashing guard column (or analytical HPLC column shown to give equivalent separations as the Biorad columns).

6. Reagents and Materials

6.1 High purity sugars for standards - glucose, xylose, arabinose, galactose, and mannose.

6.2 Second set of the high purity sugars listed above, obtained from a different source (manufacturer or lot) for preparation of calibration verification standards.

6.3 Sulfuric acid, 72% w/w (specific gravity 1.6389 at $15.6^{o}C/15.6^{o}C$).

6.4 Calcium carbonate, ACS reagent grade.

6.5 Water, HPLC grade or better, 0.2 μm filtered.

6.6 Erlenmeyer flasks, 25 mL.

6.7 Glass bottles, crimp top style, with rubber stoppers and aluminum seals to fit.

6.8 Pasteur pipettes.

6.9 pH paper (range 2-9).

6.10 0.2 μm syringe filters.

6.11 Disposable syringes, 3 mL.

6.12 Autosampler vials with crimp top seals to fit.

6.13 Volumetric pipets, class A, 20 mL.

6.14 Volumetric flasks, class A, of appropriate sizes.

6.15 Adjustable pipettors, coverings ranges of 10 to 1000 FL.

7. ES&H Considerations and Hazards

7.1 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

8. Calibration and Standardization

8.1 This analysis uses a multipoint calibration as described in the procedure.

9. Procedure

9.1 Thoroughly mix and then accurately measure out duplicate 20.0 mL portions of each sample into labeled crimp-top bottles. If the available amount of sample is limited, this procedure can by scaled back by using 10.0 mL portions. Other procedural steps must then be scaled by accordingly.

> Note: If the specific gravity of the sample is close to 1.0, the sample can be measured out by accurately weighing 20.00 g portions on a pan balance instead of by pipetting.

9.2 Dispense a separate aliquot of each sample into an Erlenmeyer flask, measure and record the pH of each sample to the nearest 0.01 pH unit.

9.3 Prepare method verification standards (MVS) by selecting representative samples to be used to determine spike recoveries. Spike an accurately measured volume of each selected sample with a known amount of the sugars of interest, such that the final concentrations of each component still falls within the linear range of the analysis. Process these spiked samples along with the rest of the samples.

9.4 Calculate the amount of 72% w/w sulfuric acid required to bring the initial acid concentration of each sample to a 4% final acid concentration (refer to the example presented in the calculation section). Swirl the sample while carefully adding the required amount of acid.

9.5 Stopper the sample bottles and crimp aluminum seals into place.

9.6 Prepare a set of sugar recovery standards (SRS) that will be taken through the complete analytical procedure to correct for losses due to the destruction of sugars during the dilute acid hydrolysis. Weigh out the required amounts of each sugar to the nearest 0.1 mg, transfer the sugars to a crimp-top bottle, and add 20.00 g HPLC grade water. A typical protocol for preparing the necessary sugar recovery standards is presented in the following table:

Sugar recovery standard (SRS)	Column	Target weights (for 20.00 mL total volume)				
		glucose	xylose	galactose	arabinose	mannose
G/X/A low	either column	25 mg	25 mg	---	25 mg	---
G/X/A mid		100 mg	100 mg	---	100 mg	---
G/X/A high		200 mg	200 mg	---	200 mg	---
G/X/Gal/A/M low	HPX-87P only	25 mg	25 mg	25 mg	25 mg	25 mg
G/X/Gal/A/M mid		100 mg	100 mg	100 mg	100 mg	100 mg
G/X/Gal/A/M high		200 mg	200 mg	200 mg	200 mg	200 mg

9.7 To each sugar recovery standard, add 697 µL of 72% sulfuric acid (refer to example in the calculation section). Stopper the bottles, label, and crimp aluminum seals into place.

9.8 Autoclave the sealed samples, method verification standards, and sugar recovery standards for one hour at 121^{o}C. After completion of the autoclave cycle, allow the hydrolyzates to cool somewhat before removing the seals and stoppers.

9.9 Neutralize the hydrolyzates with calcium carbonate to a pH of 5 to 6. Add the calcium carbonate slowly with frequent mixing to avoid problems with foaming. Monitor the pH of the solutions with pH paper and, as the pH begins to approach 4.0, slow down the rate of calcium carbonate addition and swirl frequently to avoid over-neutralizing. Keep these neutralized samples cold.

9.10 Dilute the neutralized samples as needed, so the concentration of each sugar falls within the validated range of the analytical method.

> Note: It may be useful to determine initial glucose concentrations of the samples using an alternative technique, such as YSI glucose analyzer, in order to predict whether or not the sugars in the sample will fall within the linear range of the analysis. The results from the LAP-013 analysis will also be helpful in determining if dilution will be needed. In samples such as pretreatment liquors, xylose typically is present at high concentrations, often three to five times the level of glucose. In other samples, such as the liquid fractions of fermentation samples, the levels of all the soluble sugars can be relatively low, and the samples may not require dilution.

9.11 A portion of each appropriately diluted hydrolyzate is prepared for HPLC analysis by passing the sample through a 0.2 μm syringe filter into an autosampler vial. The vial is then sealed and labeled.

9.12 Prepare a series of sugar standards in HPLC grade water at concentrations appropriate for creating a calibration curve for each of the sugars of interest. A suggested scheme for the HPX-87C column is to prepare a set of multi-component standards containing glucose, xylose, and arabinose in the range of 0.2 -12.0 mg/mL. For the HPX-87P column, galactose and mannose should be included as additional components in the standards.

> Note: Extending the range of the calibration curves beyond 12.0 mg/mL will require validation.

9.13 Prepare an independent calibration verification standard (CVS) for each set of calibration standards. This CVS should contain precisely known amounts of each sugar contained in the calibration standards, at a concentration that falls in the middle of the validated range of the calibration curve. The CVS is to be analyzed at regular intervals during the HPLC sequence and is used to verify the validity of the calibration curves throughout the HPLC run.

9.14 Analyze the calibration standards, the calibration verification standards, the hydrolyzed sugar recovery standards, the hydrolyzed samples, and the hydrolyzed method verification (spiked) samples by HPLC using an Biorad Aminex HPX-87C or HPX-87P column for glucose, xylose, and arabinose. If mannose and galactose are also to be determined, only the Biorad Aminex HPX-87P column should be used. For many analyses it is useful to run the same samples on both columns and compare the results. The following instrumental conditions are used for both the HPX-87C and the HPX-87P columns:

Sample volume: 50 µL.
Eluant: 0.2 µm filtered and degassed HPLC grade water.
Flow rate: 0.6 mL/min.
Column temperature: 85°C.
Detector: refractive index.
Run time: 20 minutes data collection plus a 15 minute post-run.

9.15 Samples containing sugar levels falling outside the validated range of the calibration curves must be rerun after appropriately diluting.

10. Calculations

10.1 For each sample and standard, calculate the volume of 72% sulfuric acid required to bring the acid concentration to a 4% final acid concentration. The molar concentration of hydrogen ions, $[H^+]$, in a sample can be calculated from its pH:

$pH = -\log[H^+]$, therefore, $[H^+] = \text{antilog}(-pH)$.

The volume of 72% sulfuric acid to be added is then calculated from the following equation:

$$V_{72\%} = \frac{[(C_{4\%} \times V_s) - (V_s \times [H^+] \times 98.08\text{g } H_2SO_4 / 2 \text{ moles } H^+)]}{C_{72\%}}$$

where:
$V_{72\%}$ is the volume of 72% acid to be added, in mL
V_s is the initial volume of sample or standard, in mL, which includes the volume of spike added (if applicable)
$C_{4\%}$ is the concentration of 4% w/w H_2SO_4, 41.0 g/L
$C_{72\%}$ is the concentration of 72% w/w H_2SO_4, 1176.3 g/L
$[H^+]$ is the concentration of hydrogen ions, in moles/L

Example #1: Calculate the amount of 72% H_2SO_4 needed to prepare a sample with a pH of 2.41 for 4% acid hydrolysis. If the pH is 2.41, then $[H^+]$=0.00389 M. Therefore:

$$\frac{[(41.0\ g/L)(20\ mL) - (20\ mL)(0.00389\ moles/L)(98.08\ g/2\ moles)]}{1176.3\ g/L} = 0.694\ mL$$

Example #2: Calculate the amount of 72% H_2SO_4 needed to prepare a sugar recovery standard for 4% acid hydrolysis. The standard itself is prepared in water with no added acid, so the pH can be assumed to be about 7. Therefore $[H^+]$=0.0000001 M, a value small enough to be ignored in the following calculation.

$$(41.0\ g/L \times 20\ mL) / 1176.3\ g/L = 0.697\ mL$$

10.2 Create a calibration curve for each sugar to be quantified using linear regression. From these curves, determine the concentration in mg/mL of the sugars present in each solution analyzed by HPLC.

10.3 Calculate and record the amount of each calibration verification standard (CVS)

$$\%\ CVS\ recovery = \frac{conc.\ detected\ by\ HPLC, mg/mL}{known\ conc.\ of\ \ standard, mg/mL} \times 100$$

recovered following HPLC analysis.

10.4 For all three sugar recovery standards (SRSs), calculate the amount of each component sugar recovered after being taken through the dilute acid hydrolysis procedure. Average the % R_{sugar} values obtained for each individual sugar and report as % $R_{ave,\ sugar}$.

$$\%\ R_{sugar} = \frac{conc.\ detected\ by\ HPLC, mg/mL}{known\ conc.\ of\ \ sugar\ before\ hydrolysis, mg/mL} \times 100$$

10.5 Use the percent hydrolyzed sugar recovery values calculated in the previous step to correct the corresponding sugar concentration values obtained by HPLC for each of the hydrolyzed samples ($C_{cor.\ sample}$) and each of the hydrolyzed spiked samples ($C_{cor.\ spiked\ sample}$), accounting for any dilution made to the sample prior to HPLC analysis.

$$C_x = \frac{C_{HPLC} \; x \; dilution \;\; factor}{\%\, R_{ave.\,sugar} \,/\, 100}$$

Where: C_{HPLC} = conc. of a sugar as determined by HPLC, mg/mL.
$\%\ R_{ave.\ sugar}$ = average recovery of a specific SRS component.
$C_x = C_{cor.\ sample}$ or $C_{cor.\ spiked\ sample}$, concentration in mg/mL of a sugar in the hydrolyzed sample or spiked sample after correction for loss on 4% hydrolysis.

10.6 Calculate and record the percent spike recoveries (% MVS recovery) for each sugar used to prepare the method verification standards analyzed by HPLC.

$$\%\ MVS\ recovery = \frac{C_{cor.\,spiked\ sample} - C_{cor.\,sample} \; x \; \dfrac{V_{sample}}{V_{final}}}{C_{spike}} \; x\,100$$

Where: $C_{cor.\ spiked\ sample}$ = concentration of the hydrolyzed spiked sample after being corrected for loss on 4% hydrolysis, in mg/mL.
$C_{cor.\ sample}$ = concentration in mg/mL of the hydrolyzed sample after correction for loss on 4% hydrolysis.
V_{sample} = volume of sample prior to spiking, in mL.
V_{final} = final volume of solution (spike plus sample), in mL.
C_{spike} = known concentration in mg/mL of the spike solution added to a sample prior to analysis.

11. Precision and Bias

11.1 In the determination of the total sugar contents of process samples, the neutralized hydrolyzates are routinely analyzed using the HPX-87P column. When these samples are known not to contain galactose and mannose, the HPX-87C column may be used instead. Based on a root mean square evaluation of duplicate data, there is a 95% certainty that the "true value" will be within the range of the average plus or minus:

- glucose 4.86% (HPX-87C) and 2.90% (HPX-87P),
- xylose 2.73% (HPX-87C) and 3.09% (HPX-87P).

Analytes at or near the detection limit could have significantly higher precision errors.

12. Quality Control

12.1 *Reported significant figures:* All results are reported in mg/mL with two decimal places. The standard deviation or relative percent difference are also to be reported.

12.2 *Replicates:* All samples are to be run in duplicate. For replicate analyses of the same sample, report the average, standard deviation, and relative percent difference (RPD).

12.3 *Relative percent difference criteria:* The maximum RPD for duplicate samples is as follows: glucose, 7.5%, and xylose, 5.0%. If the stated RPD is exceeded, the sample should be rerun. However, analytes at or near the detection limit could have significantly higher RPDs.

12.4 *Blank:* The only requirement is an instrumental blank, consisting of the HPLC grade water analyzed by HPLC in the same manner as the samples.

12.5 *Method verification standard:* This method will utilize a matrix spike as the method verification standard, as indicated in the procedure.

12.6 *Calibration verification standard:* Calibration verification standards shall be independently prepared and analyzed as described in the procedure.

12.7 *Definition of a batch:* Any number of samples which are analyzed together and recorded together. Samples within a batch must be of the same matrix. The maximum size of a batch would be limited by the equipment constraints. A batch cannot be larger than what is practical with the equipment.

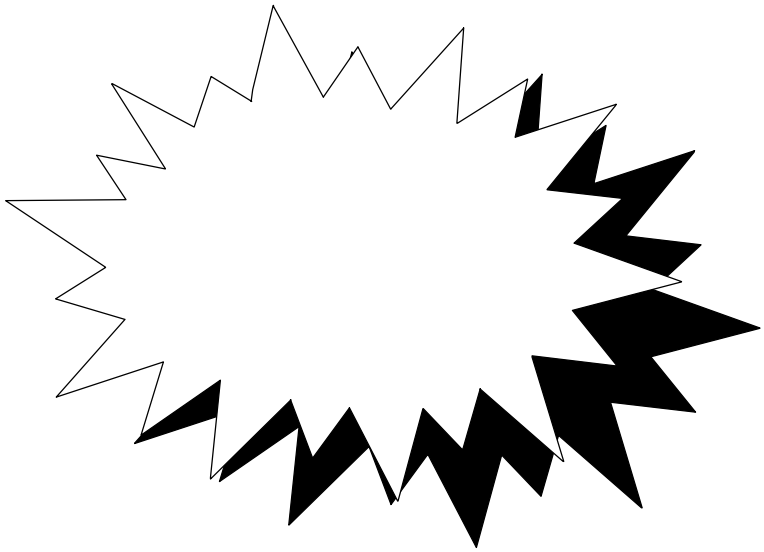
12.8 *Sample size:* 50 mL per sample.

12.9 *Sample storage:* Samples should be refrigerated.

12.10 *Standard storage:* Standards should be frozen and then shaken vigorously upon thawing.

12.11 *Standard preparation:* Standards are prepared according to instructions given in the Procedure section of this protocol.

12.12 *Control charts:* All spike recoveries and calibration verification standards are control charted.



Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-015

Procedure Title: HPLC Analysis of Liquid Fractions of Process Samples for Byproducts and Degradation Products

Author: Raymond Ruiz and Tina Ehrman

Date: 8/22/96

ISSUE DATE: 9/25/96 **SUPERSEDES:** 11/01/94

HPLC Analysis of Liquid Fractions of Process Samples for Byproducts and Degradation Products

Laboratory Analytical Procedure #015

1. Introduction

1.1 During processing of biomass samples, such as in acid pretreatment of biomass, a liquid portion is produced which may contain carbohydrate degradation products, such as HMF and furfural, as well as other components of interest, such as organic acids and sugar alcohols. These components are analyzed by HPLC with refractive index detection to determine optimal production process parameters or to monitor ongoing processes.

2. Scope

2.1 This procedure is used to determine the concentration of carbohydrate degradation products as well as selected organic acids and sugar alcohols present in the liquid fractions of biomass to ethanol process streams. These process streams include pretreatment liquors, liquid fermentation time point samples, and the liquid fraction of fermentation residues.

2.2 All analyses shall be performed according to the guidelines established in the Ethanol Project Quality Assurance Plan (QAP).

3. References

3.1 Ehrman, C.I., and M.E. Himmel. 1994. "Simultaneous Saccharification and Fermentation of Pretreated Biomass: Improving Mass Balance Closure." *Biotechnology Techniques*, 8(2):99-104.

3.2 Moore, W., and D. Johnson. 1967. *Procedures for the Chemical Analysis of Wood and Wood Products*. Madison, WI: U.S. Forest Products Laboratory, U.S. Department of Agriculture.

4. Significance and Use

4.1 This procedure is used to determine the amount of ethanol, selected organic acids and sugar alcohols, and carbohydrate degradation products (such as HMF and furfural) in the liquid fraction of biomass to ethanol process streams. Several of the compounds being measured are potential inhibitors of the process, and are therefore important to monitor.

4.2 The concentrations of these byproducts and degradation products are used in conjunction with other assays to determine the total composition of process stream samples.

5. Interferences

5.1 Arabitol coelutes with xylitol. If the sample is thought to contain arabitol, the experimentally determined xylitol concentration should be flagged as potentially being biased high due to the suspected arabitiol component.

5.2 The HPLC column used in this protocol is only partially capable of resolving the monomeric sugars of importance in biomass analysis. Glucose, xylose, and arabinose will be resolved, but galactose and mannose will coelute with xylose. If monomeric sugars are present in concentrations far exceeding the concentrations of the analytes to be quantified by this protocol, some of these analytes will appear as small humps on the shoulders of larger peaks, leading to difficulties when integrating.

5.3 In addition to the glycerol, arabitol, and xylitol, some samples may contain sorbitol. This sugar alcohol elutes about a minute earlier than xylitol on the Aminex HPX-87H column, and will appear as a peak in between the xylose and arabinose peaks.

6. Apparatus

6.1 Analytical balance, accurate to 0.1 mg.

6.2 HPLC system equipped with a refractive index detector and a Biorad Aminex HPX-87H analytical column (or equivalent) with corresponding guard column.

7. Reagents and Materials

7.1 High purity standards - including xylitol, succinic acid, lactic acid, glycerol, formic acid, acetic acid, ethanol, 5-hydroxy-2-furaldehyde (HMF), and furfural.

7.2 Second set of the high purity standards listed above, from a different source (manufacturer or lot), to be used to prepare calibration verification standards (CVS).

7.3 Sulfuric acid, concentrated, ACS reagent grade.

7.4 Water, HPLC grade or better.

7.5 0.2 μm syringe filters.

7.6 Disposable syringes, 3 mL.

7.7 Solvent filtration system equipped with 0.2 μm filter.

7.8 Autosampler vials with crimp top seals to fit.

7.9 Volumetric pipettes, class A, of appropriate sizes.

7.10 Volumetric flasks, class A, of appropriate sizes.

7.11 Adjustable pipettors, covering ranges of 10 to 1000 μL.

8. ES&H Considerations and Hazards

8.1 Sulfuric acid is corrosive and should be handled with care.

8.2 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

9. Sampling, Test Specimens and Test Units

9.1 Care must be taken to ensure a representative sample is taken for analysis.

9.2 Store sample in a sealed container to ensure the concentration of its volatile components do not change.

10. Procedure

10.1 Prepare 0.01N sulfuric acid for use as mobile phase in this analysis. In a one liter volumetric flask, add 278 μL concentrated sulfuric acid and bring to volume with HPLC grade water. Filter through a 0.2 μm filter and degas thoroughly before use.

10.2 Prepare the sample for HPLC analysis by passing it through a 0.2 μm syringe filter into an autosampler vial. Seal and label the vial. Prepare each sample in duplicate.

10.3 Prepare method verification standards (MVS) by selecting representative samples to be used to determine spike recoveries. Spike an accurately measured volume of each selected sample with a known amount of the components of interest, such that the final concentrations of each component still falls within the linear range of the analysis. Process these spiked samples along with the rest of the samples.

10.4 Prepare a series of calibration standards containing the compounds that are to be quantified, referring to Table 1 for suggested concentration ranges and special considerations. If standards are prepared outside of the suggested ranges, the new range for these calibrations curves must be validated. Since the retention times of two

pairs of components, xylitol/succinic acid and glycerol/formic acid, are so close, before standards are prepared the column should be tested to verify adequate separation and quantification can be achieved. If the separation is not adequate, either replace or regenerate the column and then confirm improved separation has been achieved. If separation is still not adequate, prepare standards without one of the components from each pair (succinic acid for the first case, formic acid for the second). An second set of standards must then be prepared containing the deleted component(s).

Table 1.

Component	Expected retention time	Suggested concentration range	*Special considerations
xylitol	11.6 min	0.2 - 6.0 mg/mL	a
succinic acid	12.0 min	0.2 - 10.0 mg/mL	a
lactic acid	13.2 min	0.2 - 12.0 mg/mL	
glycerol	14.2 min	0.2 - 8.0 mg/mL	a
formic acid	14.4 min	0.2 - 6.0 mg/mL	a
acetic acid	15.5 min	0.2 - 12.0 mg/mL	b
ethanol	22.7 min	1.0 - 15.0 mg/mL	b
HMF	29.4 min	0.02 - 5.0 mg/mL	c
furfural	42.8 min	0.02 - 5.0 mg/mL	b,c
calib. verification	--	middle of linear range	--
meth. verification	--	within linear range	d

*Special considerations:

a = confirm xylitol/succinic acid and glycerol/formic acid can be adequately separated before preparing standards.
b = samples containing volatile components must be submitted in sealed containers.
c = the linear range for HMF and furfural is limited by their solubility; when preparing a standard, add these components after the ethanol has been added to make it easier to solubilize the HMF and furfural.
d = representative sample(s) should be chosen for spiking.

10.5 Prepare an independent calibration verification standard (CVS) for each set of calibration standards, using components obtained from a source other than that used in preparing the calibration standards. This CVS must contain precisely known amounts of each compound contained in the calibration standards, at a concentration that falls in the middle of the validated range of the calibration curve (refer to Table 1). The CVS is to be analyzed after each calibration curve and at regular intervals in the HPLC sequence, bracketing groups of samples. The CVS is used to verify the quality of the calibration curve(s) throughout the HPLC run.

10.6 Analyze the calibration standards, calibration verification standards, method verification standard, and the samples by HPLC using a Biorad Aminex HPX-87H column. The following instrumental conditions are to be used for the HPX-87H column:

Sample volume: 50 µL.
Eluant: 0.2 µm filtered and degassed 0.01 N sulfuric acid.
Flow rate: 0.6 mL/min.
Column temperature: 55°C.
Detector: refractive index.
Run time: 50 minutes.

10.7 If an analyzed sample or method verification standard (spiked sample) falls outside the validated calibration range, dilute as needed and rerun the sample. The value can then be reported after correcting for dilution.

11. Calculations

11.1 Create a calibration curve for each analyte to be quantitated using linear regression. From these curves, determine the concentration in mg/mL of each component present in the samples analyzed by HPLC, correcting for dilution if required.

11.2 Calculate and record the amount of each calibration verification standard (CVS) recovered following HPLC analysis.

$$\%\ CVS\ recovered = \frac{conc.\ detected\ by\ HPLC, mg/mL}{expected\ conc.\ of\ \ standard, mg/mL}\ x100\%$$

11.3 Calculate and record the percent spike recoveries (% recovery MVS) for each component used to prepare the method verification standards analyzed by HPLC.

11.3.1 Correct the initial sample concentration for the dilution resulting from the addition of a known volume of spike solution.

$$C_{corrected} = \frac{V_{sample}}{V_{final}} \, x \, C_{sample}$$

Where: V_{sample} = volume of sample prior to spiking, in mL.
V_{final} = final volume of solution (spike plus sample), in mL.
C_{sample} = initial concentration of sample prior to spiking in mg/mL, as determined by HPLC.
$C_{corrected}$ = concentration of sample after being corrected for dilution, in mg/mL.

11.3.2 Calculate the percent recovery of the spike.

$$\% \; Recovery \; MVS = \frac{C_{actual} - C_{corrected}}{C_{spike}} \, x \, 100$$

Where: C_{actual} = actual concentration of spiked sample, as determined by HPLC, in mg/mL.
$C_{corrected}$ = concentration of sample after correcting for dilution, in mg/mL, as calculated above.
C_{spike} = known concentration of spike solution added to sample prior to analysis, in mg/mL.

12. Precision and Bias

12.1 Based on a root mean square evaluation of duplicate data, there is a 95% certainty that the "true value" will be within the range of the average plus or minus:
-glycerol, 5.52%; acetic acid, 4.58%;
-HMF, 6.15%; furfural, 5.61%;
-lactic acid, 5.85%; and succinic acid, 3.18%.

Analytes at or near the detection limit could have significantly higher precision errors.

13. Quality Control

13.1 *Reported significant figures:* All results are reported in mg/mL, with two decimal places. Also report the standard deviation and relative percent difference.

13.2 *Replicates:* All samples are run in duplicate.

13.3 *Relative percent difference criteria:* The maximum RPD for duplicate samples is as follows: glycerol, 8.8%; acetic acid, 7.1%; HMF, 9.3%; furfural, 8.9%; lactic acid, 9.4%; and succinic acid, 4.5%. If the RPD exceeds the stated value, the sample should be rerun. However, analytes at or near the detection limit could have significantly higher RPDs.

13.4 *Blank:* The only requirement is an instrument blank, consisting of the HPLC grade water analyzed by HPLC in the same manner as a sample.

13.5 *Method verification standard:* This method will utilize a matrix spike as the method verification standard, as indicated in the procedure.

13.6 *Calibration verification standard:* Calibration verification standards shall be independently prepared and analyzed as described in the procedure.

13.7 *Definition of a batch:* Any number of samples which are analyzed together and recorded together. Samples within a batch must be of the same matrix. The maximum size of a batch would be limited by the equipment constraints.

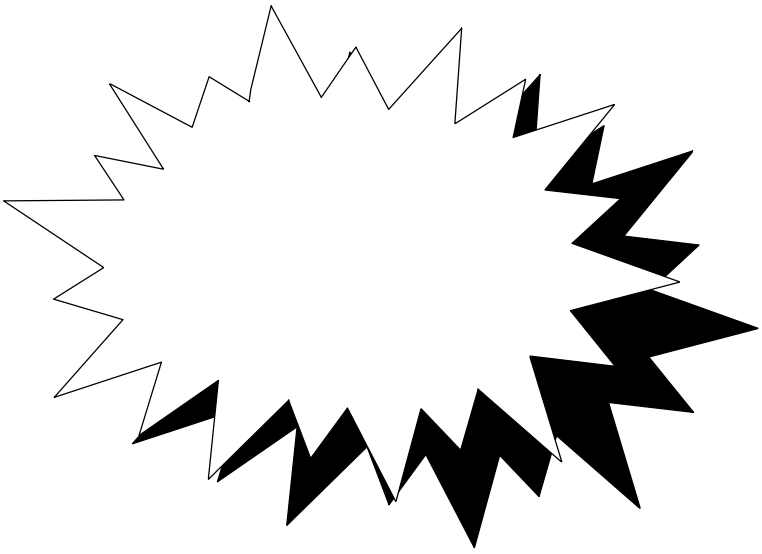
13.8 *Sample size:* 5 mL minimum.

13.9 *Sample storage:* Samples should be refrigerated. Those containing volatile components must be stored in sealed containers.

13.10 *Standard storage:* Standards should be frozen in sealed containers or vials. Shake vigorously upon thawing.

13.11 *Standard preparation:* Standards are prepared as described in the 'Procedure' section of this protocol.

13.12 *Control charts:* All spike recoveries and calibration verification standards are control charted.



Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-016

Procedure Title: Determination of Starch in Biomass Samples by Chemical Solubilization and Enzymatic Digestion

Author: Tina Ehrman

Date: 5/29/96

ISSUE DATE: 9/25/96 **SUPERSEDES:** n/a

Determination of Starch in Biomass Samples by Chemical Solubilization and Enzymatic Digestion

Laboratory Analytical Procedure #016

1. Introduction

1.1 Starch is a high molecular weight polymer consisting of glucose units linked by α-glucosidic bonds. Starch consists of two glucose polymers, amylose and amylopectin. Amylose is a linear polymer of glucose linked through α-D-1,4-glucosidic bonds while amylopectin is a branched polymer consisting of α-D-1,4-glucosidic bonds with a small number of α-D-1,6-glucosidic linkages present as interchain branch points. The relative proportions of these polymers varies with the source, but typically contains 15 to 25% amylose and 75 to 85% amylopectin. Upon hydrolysis, starch is broken down to a spectrum of higher and lower molecular weight oligosaccharides. Complete enzymatic hydrolysis yields D-glucose, which can be analyzed using an immobilized enzyme (glucose oxidase) technique.

$$\text{starch} + H_2O \xrightarrow{\text{amyloglucosidase}} \beta\text{-D-glucose (dextrose)}$$

$$\text{glucose} + O_2 \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{D-glucono-}\delta\text{-lactone}$$

1.2 Amyloglucosidase (glucoamylase) is an exoglucosidase which catalyzes the hydrolysis of both the α-D-1,6-glucosidic branch points and the predominating α-D-1,4-glucosidic linkages of starch. Amyloglucosidase removes glucose units successively from the nonreducing ends of starch chains and dextrins. The rate of hydrolysis of the α-D-1,6-glucosidic branch points is much slower than the rate for the α-D-1,4-glucosidic linkages.

2. Scope

2.1 This method covers the determination of starch in biomass samples. Sample material suitable for this procedure include hard and soft woods, herbaceous materials, agricultural residues, waste-paper, washed acid- and alkaline-pretreated biomass, and the solid fraction of fermentation residues. All results are reported relative to the 105EC oven-dried weight of the sample. In the case of extracted materials, the results may also be reported on an extractives-free basis.

2.2 This procedure is suitable for air-dried, lyophilized, and extracted biomass samples, as well as for samples that have been oven dried at a temperature of 45°C or less. The assay results will be biased slightly low for samples dried at 105°C. If sample availability is limited, it may be necessary to run this analysis on a 105°C dried sample but the results must be flagged as being biased low.

2.3 The assay is also suitable for wet samples if the particle size is known to be small and if the moisture content of the sample can be estimated accurately enough to predict the amount of sample needed to give 0.5 g of solids.

2.4 All analyses shall be preformed according to the guidelines established in the Ethanol Project Quality Assurance Plan (QAP).

3. References

3.1 NREL Ethanol Project Laboratory Analytical Procedure #001, "Standard Method for the Determination of Total Solids in Biomass".

3.2 Solvay Enzymes. 1996. *Fungal glucoamylase for Starch Hydrolysis.* Diazyme L-200 Technical Notes.

3.3 YSI Incorporated. 1994. *Determination of Cook in Extruded Cereal Products.* Application Note #319.

4. Significance and Use

4.1 The percent starch content is used in conjunction with other assays to determine the total composition of biomass samples.

5. Interferences

5.1 Interference by free glucose and cellobiose present in samples is not a problem because both glucose and cellobiose are destroyed during the NaOH solubilization step.

6. Apparatus

6.1 Analytical balance, accurate to 0.1 mg.

6.2 YSI 2700 Select Biochemistry Analyzer - equipped with a YSI 2365 dextrose membrane and YSI 2357 buffer and calibrated with YSI 2776 2.5 g/L calibrator solution.

7. Reagents and Materials

7.1 Glucose calibration verification standards, such as YSI 2.0 and 9.0 mg/mL glucose standards.

7.2 Amyloglucosidase (suggested source, Sigma A-3042).

7.3 Amylopectin (suggested source, Sigma A-7780).

7.4 Methanol, ACS reagent grade.

7.5 Hot plate or a water bath set at $90^{o} \pm 2^{o}$C.

7.6 Graduate cylinders of appropriate sizes.

7.7 100 mL, 500 mL, and 1000 mL volumetric flasks, class A.

7.8 5 and 10 mL pipets or adjustable pipettor.

7.9 Timer.

7.10 Water bath set at 40^{o} " 1^{o}C.

7.11 Serum bottles or Erlenmeyer flasks, 125 mL.

7.12. Prepared reagents:

7.12.1 2N NaOH - weigh 40 grams of sodium hydroxide pellets into a 500 mL volumetric flask. Add 300 mL of reagent grade water and mix. Cool, dilute to volume and mix.

7.12.2 2N HCl - measure 82.4 mL of concentrated hydrochloric acid and transfer to a 500 mL volumetric flask. Let cool, dilute to volume with reagent grade water and mix.

7.12.3 Acetate buffer (pH 4.2) - weigh 9.1 grams of sodium acetate into 500 mL volumetric flask. Add about 300 mL of reagent grade water and mix until all the solid is dissolved. Add 22.3 mL (23.4 grams) of glacial acetic acid. Dilute to volume with water and mix.

7.12.4 Amyloglucosidase working solution - prepare a fresh working solution of the enzyme such that it contains 60 units of activity per milliliter. If using the Sigma A-3042 amyloglucosidase, dilute the solution one hundred-fold into cold reagent grade water. Prepare daily and store in the refrigerator.

7.12.5 25% TCA - dissolve 50.0 grams of trichloracetic acid in 200 mL reagent grade water.

7.12.6 Phosphate buffer - dissolve 40 g NaH_2PO_4 and 10 g Na_2HPO_4 in reagent grade water, bring to volume in a 1000 mL volumetric flask.

8. ES&H Considerations and Hazards

8.1 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

8.2 Trichloroacetic acid is a hazardous chemical; appropriate precautions must be taken.

9. Procedure

9.1 The sample must not contain particles larger than 0.25 mm in diameter. If milling is required to reduce the particle size of the test specimen, a laboratory mill equipped with a 40 mesh, or smaller, screen should be used. If the sample size is too small to allow the use of a laboratory mill, a coffee grinder may be used instead.

Note: If pretreated samples are to be analyzed, the sample must be thoroughly washed to remove any residual acid or alkali prior to drying.

9.2 Duplicate portions of each sample must be weighed out for a total solids determination (following LAP-001) at the same time as the portions for the starch determination. If this is done later, it can introduce an error in the calculation because ground biomass can rapidly gain or lose moisture when exposed to the atmosphere. Record the average total solids value as T_{final}.

9.3 Weigh out approximately 0.50 g of sample to the nearest 0.0001 g and transfer to a 125 mL serum bottle or Erlenmeyer flask. Record as W_{sample}, the initial sample weight. Each sample must be run in duplicate, at minimum.

9.4 A standard reference material, amylopectin, is run in parallel with each batch of samples and its calculated recovery used to correct the sample results for losses due to the procedure. Weigh 0.5 g portions of amylopectin to the nearest 0.0001 g and transfer to serum bottles or Erlenmeyer flasks. Record the weight as $W_{standard}$, the initial standard reference material weight. The standard must be run in duplicate, at minimum. As with the samples, the total solids content, T_{final}, of the standard reference material must also be determined.

9.5 Add 25 mL of reagent grade water to each bottle. Swirl to ensure the sample is wetted and evenly dispersed.

> Note: A few drops of methanol may be used to prewet the sample which will aid in its dispersion once the water is added.

9.6 Add 10 mL 2N NaOH to the solution in each bottle. Place bottles on a heating unit or in a water bath preheated to 90°C. Heat for 20 minutes, swirling periodically to wet any sample that may be clinging to the side of the bottle. A glass stirring rod may be needed to break up clumps of material.

9.7 Add 10 mL 2N HCl to each bottle and swirl to mix. Cool the bottles to below 50°C.

9.8 Add 10 mL of acetate buffer to each bottle and swirl to mix.

9.9 Add 5.0 mL amyloglucosidase working solution to each bottle. Mix well and place the bottles in a 40°C water bath for 60 minutes.

9.10 After 60 minutes incubation, remove the bottles from the water bath. Immediately add 5 mL of 25% TCA to each bottle to stop hydrolysis.

9.11 Cool to room temperature and transfer each hydrolyzate to a 100 mL volumetric flask. Rinse out the bottle with small volumes of phosphate buffer and transfer all the rinses to the volumetric flask. Bring to volume with phosphate buffer and mix well.

9.12 Since the enzyme solution may contain free glucose, an enzyme blank must be run in parallel with the samples. Dilute duplicate 5.0 mL portions of the amyloglucosidase working solution to 100 mL with reagent grade water in a volumetric flask. These enzyme blanks will be analyzed in the same manner as the sample, with the averaged results used to correct the glucose contents of the samples.

9.13 The sample itself may contain free glucose, which normally would be analyzed as starch. However in this procedure the glucose, and also cellobiose, is destroyed in the NaOH solubilization step. Therefore no correction for free glucose is needed when calculating the total starch content of a sample.

9.14 Set up and calibrate the YSI as described in the manufacturer's manual using the dextrose membrane, YSI 2357 system buffer, and YSI 2776 2.5 g/L calibrator solution. Program the instrument to autocalibrate every fourth sample or every fifteen minutes, set the sample size to 25 µL, and use the following probe parameters:

Chemistry - dextrose
Units - g/L
Calibrator - 2.50 g/L
End point - 30 seconds
Cal station # - 1

9.15 Verify the calibration of the YSI using the glucose calibration verification standards before starting the run. Reverify the calibration periodically during the analysis and at the end of the run.

9.16 Measure the glucose levels in the enzyme blanks and in all the samples. The validated linear range of the instrument is 0 - 9.0 g/L dextrose. If the value reported exceeds the validated range, the hydrolyzate must be diluted appropriately and rerun.

10. Calculations

10.1 Calculate the amount of starch recovered from each analysis of the amylopectin standard reference material as follows, on a 105°C dry weight basis, and then average the recoveries:

$$\%\ Standard\ recovered = \frac{(YSI_{standard}, g/L - YSI_{enzyme\ blank}, g/L)\ x\ total\ volume, L}{standard\ weight, g, W_{standard}\ x\ \frac{\%\ total\ solids, T_{final}}{100}}\ x\ 0.9\ x\ 100\%$$

Note: Amylopectin recoveries of 93 to 95% have routinely been achieved with this protocol. Recoveries less than 90% indicate the data generated for the batch of samples should be rejected and the analysis repeated.

10.2 Calculate the amount of starch present in each sample, on a 105°C dry weight basis:

$$\%\ Starch = \frac{(YSI_{sample}, g/L - YSIsubenzyme\ blank, g/L)\ x\ total\ volume, L}{sample\ weight, g, W_{sample}\ x\ \frac{\%\ total\ solids, T_{final}}{100}}\ x\ 0.9\ x\ 100\%$$

Note: The factor 0.9 converts grams of glucose to grams of the anhydrosugar (starch, in this case). The factor can be calculated by dividing the molecular weight of glucose less one molecule of water (180 - 18) by the molecular weight of glucose.

10.3 The calculated percent starch in each sample can be corrected for assay losses using the percent recovery of the standard reference material, amylopectin, as follows:

$$\%\ Starch, corrected = \frac{\%\ starch}{average\ \%\ standard\ recovered}\ x\ 100\%$$

11. Report

11.1 Report the percent starch present in the sample, to two decimal places, on a 105°C dry weight basis.

11.2 For replicate analyses of the same sample, report the average, standard deviation, and relative percent difference (RPD).

12. Precision and Bias

12.1 Data obtained by replicate testing of a corn stover in one laboratory gave a standard deviation of 0.28% and a CV of 1.31%. Data obtained by replicate testing of a amylopectin reference material in three laboratories gave a standard deviation of 0.58% and a CV of 4.61%.

12.2 This procedure has been validated for materials which have been air-dried, lyophilized, or oven dried at a temperature of 45°C or less. The assay results will be biased slightly low for samples dried at 105°C. If sample availability is limited, it may be necessary to run this analysis on a 105°C dried sample but the results must be flagged.

13. Quality Control

13.1 *Reported significant figures:* Report the percentage of starch present in the sample to two decimal places, on a 105°C dry weight basis, or extractives-free basis. Cite the basis used in the calculation.

13.2 *Replicates:* At minimum, all samples and the method verification standard are to be analyzed in duplicate.

13.3 *Blank:* As described in the "Procedure" section, enzyme blanks are prepared in duplicate and analyzed in the same manner as the samples.

13.4 *Relative percent difference criteria:* The RPD must be less than 8.0%. If the RPD is too large, the sample must rerun.

13.5 *Method verification standard:* In this procedure, amylopectin is used as both a method verification standard and as a means for estimating and correcting for assay losses. At minimum, the amylopectin must be run in duplicate with every batch of samples. If the recovery of amylopectin is less than 90%, the data generated for the whole batch of samples must be rejected and the analysis repeated.

13.6 *Calibration verification standard:* Calibration verification standards may be purchased from YSI or independently prepared. The CVSs are analyzed periodically through the YSI run as described in the "Procedures" section.

13.7 *Sample size:* A minimum of 3.6 grams of sample (on a dry weight basis) are required for duplicate analyses, which includes both the starch and the total solids assays. If there is insufficient sample, the result will be flagged and the lack of precision noted.

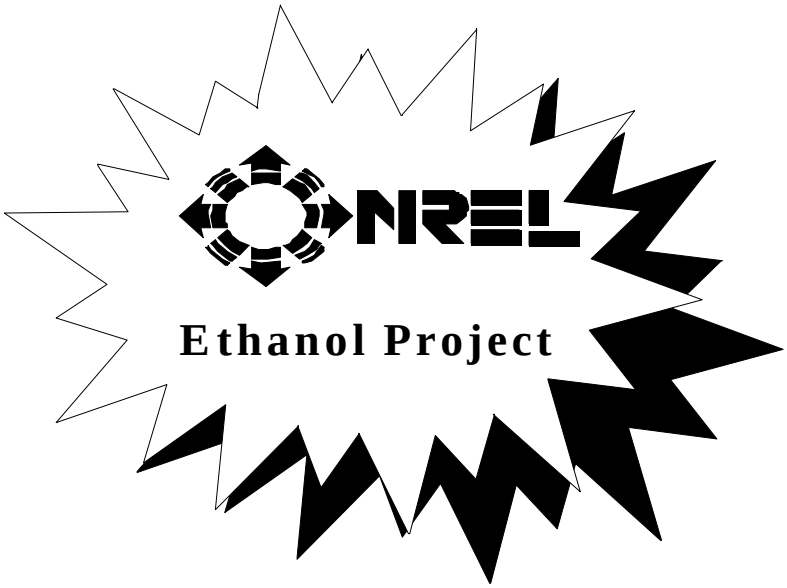
13.8 *Sample storage:* Dried samples shall be stored in an airtight container at room temperature. Samples with moisture contents greater than 10% shall be stored in an airtight container and refrigerated for not longer than one week. If longer periods of storage are required, these samples must be stored frozen.

13.9 *Standard storage:* YSI standards should be stored refrigerated and should not exceed the manufacturer's stated expiration data.

13.10 *Standard preparation:* Purchase the YSI 2776 2.5 g/L calibrator solution from YSI.

13.11 *Definition of a batch:* Any number of samples which are analyzed and recorded together. The maximum size of a batch would be limited by the equipment constraints.

13.12 *Control charts:* The result of each replicate analysis of the method verification standard (amylopectin) is recorded along with the average, RPD, and a laboratory book/page reference. The average value obtained for each analysis of the method verification standards is to be control charted.



Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-017

Procedure Title: Determination of O-Acyl Groups in Biomass by High Performance Liquid Chromatography

Author: Tina Ehrman and Raymond Ruiz

Date: 07/31/98

ISSUE DATE: 07-31-98 **SUPERSEDES:** n/a

Determination of O-Acyl Groups in Biomass by High Performance Liquid Chromatography

Laboratory Analytical Procedure #017

1. Introduction

1.1 Aliphatic groups in wood and herbaceous feedstocks are acetyl and formyl groups which can be combined as O-acyl groups with the polysaccharide portion. A number of different approaches can be used to analyze for acetyl, including acid hydrolysis, saponification, transesterification, spectrophotometirc, and aminolysis. Acid hydrolysis was selected as the approach of choice since the hydrolyzate produced during the routine compositional analysis of cellulosic samples could also be used in the analysis of O-acyl groups. In this approach, dilute acid is used to split O-acyl groups from the polysaccharides. The resulting acetic and formic acids are then quantified by HPLC.

2. Scope

2.1 This procedure describes a HPLC method for determining the amount of acetyl and formyl groups cleaved upon hydrolysis of a biomass sample. The protocol utilizes the hydrolyzate generated by LAP-002, "Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography". Special handling during the post-autoclave steps of LAP-002 is required to ensure the volatile components are not lost.

2.2 Sample material suitable for this procedure include hard and soft woods, herbaceous materials (such as switchgrass and sericea), agricultural residues (such as corn stover, wheat straw, and bagasse), and waste-paper (such as office waste, boxboard, and newsprint). Pretreated biomass may also be analyzed by this method, although most pretreatment conditions will have already removed the acetyl and formyl groups. All results are reported relative to the 105EC oven-dried weight of the sample. In the case of extracted materials, the results may also be reported on an extractives-free basis.

2.3 All analyses shall be performed according to the guidelines established in the Ethanol Project Quality Assurance Plan (QAP).

3. References

3.1 Solar, R., F. Kacik, and I. Melcer. 1987. Simple Semimicro Method for the Determination of O-Acetyl Groups in Wood and Related Materials. Nordic Pulp and Paper Research Journal, 4:139-141.

3.2 Ethanol Project Laboratory Analytical Procedure #001, "Standard Method for the Determination of Total Solids in Biomass".

3.3 Ethanol Project Laboratory Analytical Procedure #002, "Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography".

3.4 Ethanol Project Laboratory Analytical Procedure #003, "Determination of Acid-Insoluble Lignin in Biomass".

3.5 Ethanol Project Laboratory Analytical Procedure #004, "Determination of Acid-Soluble Lignin in Biomass".

3.6 NREL Ethanol Project Laboratory Analytical Procedure #010, "Standard Method for the Determination of Extractives in Biomass".

4. Terminology

4.1 Prepared Biomass - Biomass that has been prepared by lyophilization, oven drying, air drying, and in some instances by extraction, to reduce the moisture content of the sample so it is suitable for O-acetyl group analysis.

4.2 Oven-Dried Weight - The moisture-free weight of a biomass sample as determined by LAP-001, "Standard Method for Determination of Total Solids in Biomass".

5. Significance and Use

5.1 The percent acetyl and formyl group contents are used in conjunction with other assays to determine the total composition of biomass samples.

6. Interferences

6.1 Formic acid is produced not only from the cleaving of O-formyl groups but also from the hydrolysis of HMF. The amount of levulinic acid present may be used as a rough indicator of the source of the formic acid, since levulinic acid is produced in equal molar quantities with formic acid when HMF is hydrolyzed.

7. Apparatus

7.1 Hewlett Packard Model 1090 HPLC, or equivalent, with refractive index detector.

7.2 HPLC column, BioRad Aminex@ HPX-87H (or equivalent).

7.3 Guard column, cartridges appropriate for the HPLC column used.

7.4 Analytical balance readable to 0.1 mg.

8. Reagents and Materials

8.1 Reagents

8.1.1 High purity chemicals for standards (98%+) - two sets of acetic acid, formic acid, and levulinic acid from different lots or manufacturers.

8.1.2 0.01 N H_2SO_4.

8.1.3 Water, 18 megohm deionized.

8.2 Materials

8.2.1 Disposable nylon syringe filters, 0.2 μm.

8.2.2 Disposable syringes, 3 mL.

8.2.3 Autosampler vials, with crimp top seals to fit.

9. ES&H Considerations and Hazards

9.1 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

9.2 Use caution when handling hot glass bottles after the autoclave step, as they may have become pressurized.

10. Sampling, Test Specimens and Test Units

10.1 The hydrolyzates generated as part of LAP-002 or LAP-003 are to be utilized in this method. Sample weight, total solid content, and extractive content (if needed) are determined as specified in the LAP-002 or LAP-003 protocols. The unused portion of the hydrolyzate can be taken through the LAP-002 and LAP-003 as desired.

11. Procedure

11.1 Upon removal of the hydrolyzed biomass samples from the autoclave (as part of the LAP-002 or LAP-003 procedure), allow the contents of the bottles to cool for 20 minutes at room temperature. Swirl the contents of each bottle vigorously and allow to cool further, until the contents are at room temperature and the solids have settled.

11.2 Working with one bottle at a time, remove the seal and stopper, taking care not to stir up any solids which have settled to the bottom.

11.3 Using a disposable syringe, immediately remove about 1.5 mL of sample, again taking care not to disturb the settled solids. Quickly pass this aliquot through a 0.2 μm filter into an autosampler vial. Immediately cap the vial.

11.4 Prepare a series of calibration standards in deionized water at concentrations appropriate for creating a calibration curve for each component of interest. A suggested scheme for the HPX-87H column is to prepare a set of multi-component standards containing acetic acid, formic acid, and levulinic acid in the range of 0.02 to 0.5 mg/mL.

11.5 Prepare an independent calibration verification standard (CVS) using chemicals obtained from a source other than that used in preparing the calibration standards. The CVS must contain precisely known amounts of each component contained in the calibration standards, at a concentration that falls in the middle of the validated range of the calibration curve. The CVS is to be analyzed after each calibration curve and at regular intervals in the HPLC sequence, bracketing groups of samples. The CVS is used to verify the quality of the calibration curve(s) throughout the HPLC run.

11.6 Analyze the calibration standards, the CVS, the MVS, and the samples by HPLC using a Biorad Aminex@ HPX-87H column. The following instrumental conditions are used:

Sample volume: 50 FL.
Eluant: 0.2 μm filtered and degassed 0.01 N H_2SO_4.
Flow rate: 0.6 mL/min.
Column temperature: 55°C.
Detector: refractive index.
Run time: 20 minutes (refer to precautionary note which follows).

Note: The hydrolyzates being tested will contain low levels of HMF and/or furfural. These components will appear as peaks in the chromatogram of the following sample. It is important to verify the HMF and furfural peaks are not interfering with the peaks of interest. With the instrument set up used to develop this method, use of a 20 minute run time resulted in the HMF peak appearing at about 10 minutes into the following chromatogram, and the furfural peak appearing at about 19 minutes. Neither peak interfered with the analytes of interest.

12. Calculations

12.1 Create a calibration curve by linear regression analysis for each component to be quantified. From these curves, determine the concentration in mg/mL of the organic acids present in each solution analyzed by HPLC.

12.2 For lyophilized, air dried, or oven dried samples, or for samples requiring no preparation, calculate the percentage of each organic acid present in the sample on an as received 105°C dry weight basis follows:

$$\%\ Analyte = \frac{C\ x\ \frac{1\ g}{1000\ mg}\ x\ V_F}{W_1\ x\ \frac{\%T_{as\ received}}{\%T_{prep}}}\ x\ 100\% = \frac{C\ x\ \frac{1\ g}{1000\ mg}\ x\ V_F}{W_1\ x\ \frac{\%T_{final}}{100\%}}\ x\ 100\%$$

Where: W_1 = initial weight of sample, in grams
V_F = volume of filtrate, 87.0 mL
C = concentration of component in hydrolyzed sample in mg/mL
$\%T_{as\ received}$ = % total solids content of the original sample (prior to any preparation steps) on a 105°C dry weight basis, as determined by the LAP-001
$\%T_{prep}$ = % total solids content of the sample as determined during the preparation of the sample for analysis (by lyophilization, oven-drying, or air drying)
$\%T_{final}$ = % total solids content of the prepared sample used in this analysis, on a 105°C dry weight basis, as determined by the LAP-001

Note: If the sample used in the analysis required no preparation (analyzed as received), then $\%T_{prep} = 100\%$ and $\%T_{final} = \%T_{as\ received}$.

12.3 If the biomass was prepared according to the "Standard Method for the Determination of Ethanol Extractives in Biomass" (LAP-010), first calculate the percentage of each organic acid on an extractives-free 105°C dry weight basis and then correct this value to an as received (whole sample) 105°C dry weight basis.

12.3.1 Calculate the percentage of each component on an extractives-free basis as follows:

$$\%\ Analyte_{extractives\text{-}free} = \frac{C \times \frac{1\ g}{1000\ mg} \times V_F}{W_1 \times \frac{\%T_{final}}{100\%}} \times 100\%$$

Where: C = concentration of component in hydrolyzed sample in mg/mL
V_F = volume of filtrate, 87.0 mL
W_1 = initial weight of extracted sample, in grams
$\%T_{final}$ = % total solids content of the prepared sample used in this analysis (in this case, extracted sample), on a 105°C dry weight basis, as determined by LAP-001

12.3.2 Correct the % organic acid value on an extractives-free basis, calculated above, to an as received (whole sample) 105EC dry weight basis as follows:

$$\%\ Analyte_{whole\ sample} = \%\ Analyte_{extractives\text{-}free} \times \frac{(100\% - \%\ extractives)}{100\%}$$

Where: % $Analyte_{extractives\text{-}free}$ = % analyte on an extractives-free 105°C dry weight basis, as determined in the previous step
% extractives = % extractives in the extracted sample as described in the Standard Method for the Determination of Extractives in Biomass (LAP-010)

13. Report

13.1 Report the percent of each analyte to two decimal places, on a whole sample 105°C dry weight basis or on an extractives-free basis. Cite the basis used in the report.

13.2 For replicate analyses of the same sample, report the average, standard deviation, and relative percent difference (RPD).

14. Precision and Bias

14.1 Data obtained by replicate testing of a hybrid poplar in two laboratories, using an HPX-87H column, gave a standard deviation in acetic-acid content of 0.08% and a CV% of 1.77%.

15. Quality Control

15.1 *Reported significant figures:* Report the percentage of each analyte present in the hydrolyzed sample to two decimal places, on a whole sample 105°C dry weight basis or on an extractives-free basis. Cite the basis used in the calculation.

15.2 *Replicates:* At minimum, all samples and the method verification standard are to be analyzed in duplicate.

15.3 *Blank:* The only requirement is a reagent blank specified in LAP-002 and LAP-003, which starts out as an empty 16x100 mm test tube (ie, no sample) which is taken through all the procedural steps.

15.4 *Relative percent difference criteria:* The RPD for acetic-acid must be less than 2.75%. If the RPD is too large, the sample must be rerun.

15.5 *Method verification standard:* A method verification standard must be run in duplicate with every batch. This method utilizes a well characterized standard material suitable for analysis. For example, NIST 8492 (*Populus deltoides*) is used as the MVS in O-acyl analysis of hardwoods.

15.6 *Calibration verification standard:* Calibration verification standards shall be independently prepared and analyzed as described in section 11.5 of this procedure.

15.7 *Sample size:* As specified in LAP-002 and LAP-003, a minimum of 0.6 grams of sample (on a dry weight basis) are required for duplicate analyses. If there is insufficient sample, the result will be flagged and the lack of precision will be noted.

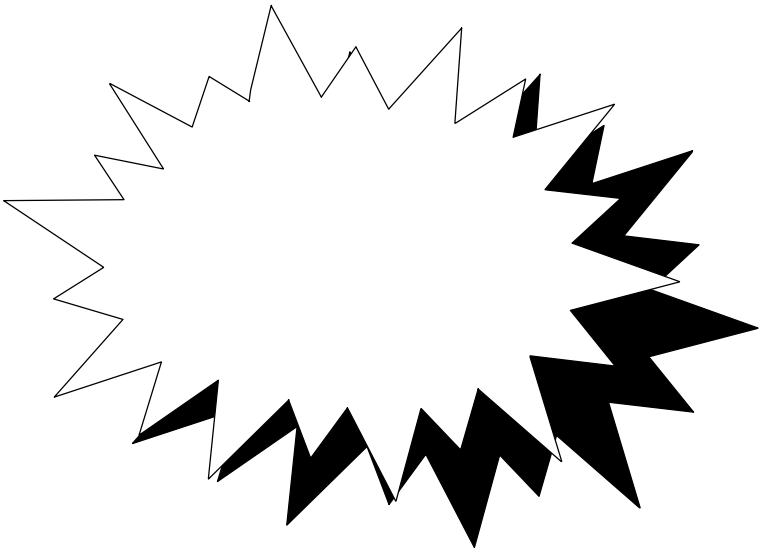
15.8 *Sample storage:* Samples shall be stored in an airtight container and refrigerated.

15.9 *Standard storage:* Standards should be kept frozen in airtight vials or test tubes. Vortex the standards vigorously upon thawing to ensure thorough mixing.

15.10 *Standard preparation:* Standards are prepared as described in section 11.4 of this method.

15.11 *Definition of a batch:* Any number of samples which are analyzed and recorded together. The maximum size of a batch would be limited by the equipment constraints. A batch cannot be larger than what is practical with the equipment.

15.12 *Control charts:* The result of each replicate analysis of the method verification standard is recorded along with the average, RPD, and a laboratory book/page reference. The average value obtained for each analysis of the method verification standards is to be control charted.



Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-018

Procedure Title: **Determination of Insoluble Solids of Pretreated Biomass Material**

Author: Fannie Posey Eddy, Jody Okafor, Candic Roberson

Date: **9/23/98**

ISSUE DATE:9/18 **SUPERSEDES:**

Determination of Insoluble Solids in Pretreated Biomass

Laboratory Analytical Procedure #018

1. Introduction

1.1 Pretreated biomass samples are composed of water-soluble and water insoluble components. These two fractions are analyzed separately. Analytical results are then mathematically recombined in the computation of the mass balance on the pretreatment process. To separate the water soluble portion from the sample a thorough water extraction is performed leaving a portion of solid material called the insoluble solids fraction of the sample. This method describes two reliable procedures for determining both the percent insoluble solids and percent fraction insoluble solids in a sample of hydrolyzate slurry from pretreated biomass.

2. Scope

2.1 This procedure is intended to determine the percentage of water insoluble solids in a pretreated biomass sample after all soluble components have been extracted with aggressive water washing.

2.2 All analyses shall be performed according to guidelines established by the Ethanol Quality Insurance Plan.

3. References

3.1 NREL Ethanol Project Laboratory Analytical Procedure #012, "Standard Test Method for Moisture, Total Solids, and Total Dissolved Solids in Biomass Slurry and Liquid Process Samples.

4. Terminology

4.1 **Pretreated Biomass**-Biomass which has been chemically and/or thermally altered to change the structural composition.

4.2 **Hydrolyzate slurry**-The liquid and solid material in a sample resulting from biomass pretreatment.

4.3 **Hydrolyzate Liquor**- Liquid portion of hydrolyzate slurry.

4.4 **Washed Solids-**Water insoluble portion of hydrolyzate slurry.

4.5 **Pressate**-Liquid product from pretreated biomass pressed via centripetal force, manual or hydraulic pressure.

4.6 **Filtrate**-Hydrolyzate liquid product from hydrolyzate slurry which has been placed in a Buchner funnel and vacuum filtered.

4.7 **Oven Dried Weight**-The moisture-free weight of a biomass sample as determined by Lap-001, Standard Method for Determination of Total Solids in Biomass.

4.8 **Insoluble Solids (IS)-**The oven dried weight of water insoluble solids divided by the weight of whole hydrolyzate slurry sample (as received).

4.9 **Fraction Insoluble Solids (FIS)-**The oven dried weight of water insoluble solids divided by the *oven dried weight* of the whole hydrolyzate slurry.

5. Significance and Use

5.1 The percent insoluble solids and the percent fraction insoluble solids are used to combine the liquid and solid compositions of the pretreated biomass in the mass balance determination.

6. Interference

6.1 Technique is critical to minimizing the amount of material lost during the wash steps. Care should be taken when separating the liquid from the solids or results will not meet standard quality assurance requirements.

6.2 Hydrolyzate slurries separate quickly. Special attention is recommended when taking samples .To obtain a representative sample thoroughly mix prior to taking a sample.

7. Apparatus *Procedure A (Centrifugation)*

7.1 Analytical balance readable to 0.1 mg.

7.2 Convection ovens with temperature control to 45 °C ± 3 and 105 °C ± 3.

7.3 Analytical balance readable to 0.01g.

7.4 Desiccator.

7.5 Centrifuge refrigerated to 4°C and rotor specified to hold 300 ml. capacity bottles and rated at least 9,000 rpm.

7.6 Aluminum foil weighing dish.

7.7 Centrifugation bottles with wide opening, caps with seals and 300 ml capacity/reservoir.

7.8 Biochemical analyzer (YSI) for measurement of glucose (optional).

7.9 Eppendorf microcentrifuge tubes and transfer pipettes (optional).

8. Apparatus *Procedure B (Filtration)*

8.1 Whatman GF/D 2.7um glass microfiber FilterCup (1600R823) with FilterCup stem (1600R900). An alternate is Buchner funnels (two-part, polypropylene) with GF/D glass microfiber filter paper.

8.2 Glass microfiber filter paper sized for the Buchner funnel chosen.

8.3 1000 ml vacuum flask.

8.4 Filtration set-up including vacuum source and vacuum adapters for Buchner funnels.

8.5 Items 1-4 as described in section 7.

8.6 Teflon coated spatulas.

9. Reagents and Materials

9.1 pH paper (range 2-9).

9.2 Electronic pipette and disposable pipettes in the range of 25 ml.

9.3 2,000 ml flask or beaker.

9.4 YSI dextrose standards 2.0 g/L, 9.0 g/L (optional).

9.5 Water 18 megaohm deionized.

10. ES&H Considerations and Hazards

10.1 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

11. Procedure A *(Centrifugation)*

11.1 Record weight of *centrifuge bottle and cap* to nearest the 0.01g on an analytical balance and tare.

11.2 Add 25-50 grams of sample. Record *weight of sample as received* to the nearest 0.01g.

11.3 Add 175-200 grams of deionized water. Shake vigorously for 60 seconds. (Note: If collecting liquor for further analysis, do so prior to this step).

11.4 Cap and spin in centrifuge at 4°C for 20 minutes at 9,000 rpm.

11.5 Carefully remove bottles from centrifuge to minimize disturbance of sample pellet.

11.6 Remove liquid portion by decanting without disturbing sample pellet. Use electronic or manual pipetter to remove small amounts of liquid until small traces are left.

11.7 Repeat steps 10.3-10.6 three more times or until sample pH is at least 4.5-7.0. (For some applications it may be necessary to test the amount of glucose present by biochemistry analyser in determining if another wash is necessary. Glucose remaining in the last wash should not exceed more that 0.05 g/L.)

11.8 Record weight of *bottle, cap and sample* to the nearest 0.01g. Then subtract weight of bottle and cap from this recorded weight to caluculate and record the *weight of washed*

sample.

11.9 Perform total solids on the as received and washed material as described in Lap-001.

11.10 Store remaining sample in refrigerator or freezer as appropriate to maintain sample integrity.

12. Procedure B *(Filtration)*

12.1 Record weight of buchner funnel filter cup with glass filter paper and record to the nearest 0.01g on analytical balance and tare.

12.2 Add 25-50 grams of sample. (Record *weight of sample as received*).

12.3 Gradually pour 200-250 ml of deionized water. Stir gently to mix sample. Allow vacuum filtration to proceed slowly.

12.4 Use pH sticks to test the pH of the filtrate and sample.Repeat steps 12.2-12.3 three more times or until sample pH is at least 4.5-7.0. (For some applications it may be necessary to test the amount of glucose present by biochemistry analyser in determining if another wash is necessary. Glucose remaining in the last wash should not exceed more than 0.5 g/L.)

12.5 Let process sit under vacuum for 5 minutes to remove excess water. Remove filter cup and place on paper towel to allow water accumulation on filter cup bottom to escape.

12.6 Weigh filter cup with glass fiber filter paper and sample. Record weight. Subtract the weight of the filter cup and filter to get the *wet weight of the washed sample*. Then place in 105°C oven for overnight.. *(If alternate Buchner funnel is used check with manufactuer for temperature limitations. Use 45°C as oven temperature if necessary and dry to a constant weight.)*

12.7 Remove filter cup with sample from oven and place in desiccator for five minutes. Weigh filter cup + filter + sample and record. Subtract weight of filter cup and filter paper to get dry sample weight. Record *dry weight of washed sample* to the nearest 0.01 grams.

12.8 Perform total solids on the as received material as described in Lap-001. The % total

solids on the washed sample is calculated from *dry weight of washed sample* in step 12.7.

12.9 Discard or store sample as necessary.

13. Calculations

13.1 Calculate the percent insoluble solids and fraction insoluble solids for each sample on a percent dry weight basis. Total solids by LAP-001, on the as received and the washed sample will be necessary for each procedure. However, in *Procedure B (Filtration)* the percent total solids of the washed material is calculated based on the whole sample weight.

Procedure A (Centrifugation)

Weight of washed sample = (Weight of bottle, cap, & washed sample) – (Weight of bottle and cap)

$$\text{Dry Weight of Washed sample} = \frac{\text{(Weight of washed sample) (\% Total solids of washed sample)}}{100}$$

$$\textbf{\% Insoluble Solids} = \frac{\text{Dry weight of washed sample}}{\text{Weight of sample as received}} \times 100$$

$$\text{Dry Weight of Sample As Received} = \frac{\text{(Weight of Sample As Received) X (\% Total Solids As Received)}}{100}$$

$$\textbf{\% Fraction Insoluble Solids} = \frac{\text{Dry Weight of Washed sample}}{\text{Dry weight of sample as received}} \times 100$$

13.2 Percent insoluble solids and percent fraction insoluble solids can be calculated as described in Procedure A provided the percent total solids are calculated as demonstrated below. The percent total solids of the washed sample are based on the entire sample that is washed and dried in the filter cup.

Procedure B (Filtration)

Weight of washed sample = (Weight of filter cup, filter, & washed sample) – (Weight of filter cup & filter)

***% Total Solids on As Received Sample** = As described in LAP 001*

$$\textbf{\% Total Solids on Washed Sample} = \frac{\text{Dry Weight of the Washed Sample}}{\text{Wet Weight of Washed Sample}} \times 100$$

14. Report

14.1 Report the result as a percentage with two decimal places.

14.2 For replicate analyses of the same sample, report the average, standard deviation, and relative percentage difference.

15. Precision and Bias

15.1 Analysis in one laboratory of a method verification standard showed a sample recovery of 95.6% (IS) and 100.48 (FIS) with a coefficient of variation of 1.28% (IS) and 1.52% (FIS) for Procedure B (Filtration). In Procedure A (Centrifugation) the recovery values were 96.49% (IS) and 100.09 (FIS) with a coefficient of variation of 1.56% (IS) and 1.78% (FIS). Estimation of standard deviation from duplicate numbers was 2.46 (IS) and .03(FIS) for Procedure A(centrifugation) and 0.09(IS) and 0.006)(FIS) for Procedure B(filtration).

15.2 Statistical analysis for several different types of biomass material gave an estimation of standard deviation of 0.59(IS) for Procedure A and 0.10(IS) Procedure B. Statistical analysis comparing both procedures indicated no significant difference between the two methods, assuming a 95% confidence level.

16. Quality Control

16.1 *Reported significant figures:* Report all data to two significant figures.

16.2 *Replicates:* Run all samples in duplicate or triplicate if time permits.

16.3 *Blank:* Not applicable.

16.4 *Relative percent difference criteria:* % RPD should not exceed 6%.

16.5 *Method verification standard:* A method verification standard should be run with each sample set. Solka Floc can be processed as the MVS by both procedures. Use 10-15g sample size when selecting Solka Floc as the MVS.

16.6 *Calibration verification standard:* Not Applicable.

16.7 *Sample size*: A minimum of 25-50 grams should be processed in these procedures. It is possible scale up the process depending on equipment and time constraints.

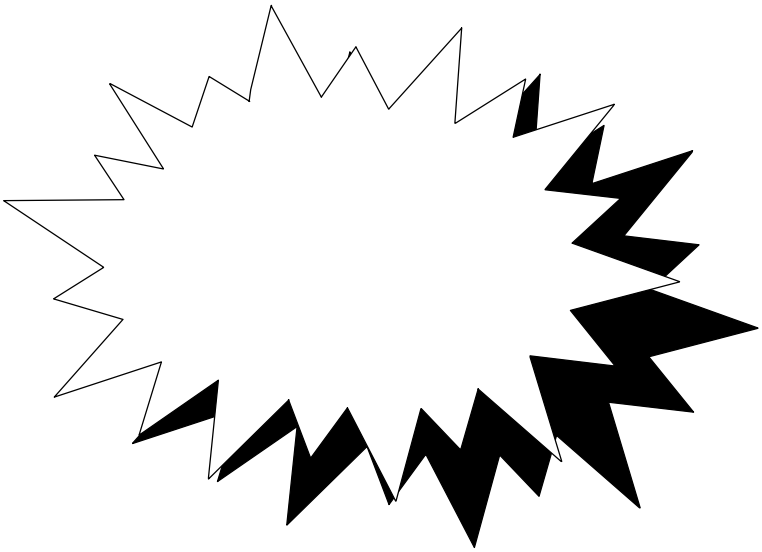
16.8 *Sample storage* Samples shall be stored in the refrigerator.

16.9 *Standard storage: Not Applicable.*

16.10 *Standard preparation:* Not Applicable.

16.11 *Definition of a sample set*: Any number of samples analyzed together and recorded together within the limitation of instrumentation and time requirements.

16.12 *Control charts:* A control chart should be kept for the method verification standard.



Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-018

Procedure Title: **Determination of Insoluble Solids of Pretreated Biomass Material**

Author: Fannie Posey Eddy, Jody Okafor, Candic Roberson

Date: **9/23/98**

ISSUE DATE:9/18 **SUPERSEDES:**

Determination of Insoluble Solids in Pretreated Biomass

Laboratory Analytical Procedure #018

1. Introduction

1.1 Pretreated biomass samples are composed of water-soluble and water insoluble components. These two fractions are analyzed separately. Analytical results are then mathematically recombined in the computation of the mass balance on the pretreatment process. To separate the water soluble portion from the sample a thorough water extraction is performed leaving a portion of solid material called the insoluble solids fraction of the sample. This method describes two reliable procedures for determining both the percent insoluble solids and percent fraction insoluble solids in a sample of hydrolyzate slurry from pretreated biomass.

2. Scope

2.1 This procedure is intended to determine the percentage of water insoluble solids in a pretreated biomass sample after all soluble components have been extracted with aggressive water washing.

2.2 All analyses shall be performed according to guidelines established by the Ethanol Quality Insurance Plan.

3. References

3.1 NREL Ethanol Project Laboratory Analytical Procedure #012, "Standard Test Method for Moisture, Total Solids, and Total Dissolved Solids in Biomass Slurry and Liquid Process Samples.

4. Terminology

4.1 **Pretreated Biomass**-Biomass which has been chemically and/or thermally altered to change the structural composition.

4.2 **Hydrolyzate slurry**-The liquid and solid material in a sample resulting from biomass pretreatment.

4.3 **Hydrolyzate Liquor**- Liquid portion of hydrolyzate slurry.

4.4 **Washed Solids-**Water insoluble portion of hydrolyzate slurry.

4.5 **Pressate**-Liquid product from pretreated biomass pressed via centripetal force, manual or hydraulic pressure.

4.6 **Filtrate**-Hydrolyzate liquid product from hydrolyzate slurry which has been placed in a Buchner funnel and vacuum filtered.

4.7 **Oven Dried Weight**-The moisture-free weight of a biomass sample as determined by Lap-001, Standard Method for Determination of Total Solids in Biomass.

4.8 **Insoluble Solids (IS)-**The oven dried weight of water insoluble solids divided by the weight of whole hydrolyzate slurry sample (as received).

4.9 **Fraction Insoluble Solids (FIS)-**The oven dried weight of water insoluble solids divided by the *oven dried weight* of the whole hydrolyzate slurry.

5. Significance and Use

5.1 The percent insoluble solids and the percent fraction insoluble solids are used to combine the liquid and solid compositions of the pretreated biomass in the mass balance determination.

6. Interference

6.1 Technique is critical to minimizing the amount of material lost during the wash steps. Care should be taken when separating the liquid from the solids or results will not meet standard quality assurance requirements.

6.2 Hydrolyzate slurries separate quickly. Special attention is recommended when taking samples .To obtain a representative sample thoroughly mix prior to taking a sample.

7. Apparatus *Procedure A (Centrifugation)*

7.1 Analytical balance readable to 0.1 mg.

7.2 Convection ovens with temperature control to 45 °C ± 3 and 105 °C ± 3.

7.3 Analytical balance readable to 0.01g.

7.4 Desiccator.

7.5 Centrifuge refrigerated to 4°C and rotor specified to hold 300 ml. capacity bottles and rated at least 9,000 rpm.

7.6 Aluminum foil weighing dish.

7.7 Centrifugation bottles with wide opening, caps with seals and 300 ml capacity/reservoir.

7.8 Biochemical analyzer (YSI) for measurement of glucose (optional).

7.9 Eppendorf microcentrifuge tubes and transfer pipettes (optional).

8. Apparatus *Procedure B (Filtration)*

8.1 Whatman GF/D 2.7um glass microfiber FilterCup (1600R823) with FilterCup stem (1600R900). An alternate is Buchner funnels (two-part, polypropylene) with GF/D glass microfiber filter paper.

8.2 Glass microfiber filter paper sized for the Buchner funnel chosen.

8.3 1000 ml vacuum flask.

8.4 Filtration set-up including vacuum source and vacuum adapters for Buchner funnels.

8.5 Items 1-4 as described in section 7.

8.6 Teflon coated spatulas.

9. Reagents and Materials

9.1 pH paper (range 2-9).

9.2 Electronic pipette and disposable pipettes in the range of 25 ml.

9.3 2,000 ml flask or beaker.

9.4 YSI dextrose standards 2.0 g/L, 9.0 g/L (optional).

9.5 Water 18 megaohm deionized.

10. ES&H Considerations and Hazards

10.1 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

11. Procedure A *(Centrifugation)*

11.1 Record weight of *centrifuge bottle and cap* to nearest the 0.01g on an analytical balance and tare.

11.2 Add 25-50 grams of sample. Record *weight of sample as received* to the nearest 0.01g.

11.3 Add 175-200 grams of deionized water. Shake vigorously for 60 seconds. (Note: If collecting liquor for further analysis, do so prior to this step).

11.4 Cap and spin in centrifuge at 4°C for 20 minutes at 9,000 rpm.

11.5 Carefully remove bottles from centrifuge to minimize disturbance of sample pellet.

11.6 Remove liquid portion by decanting without disturbing sample pellet. Use electronic or manual pipetter to remove small amounts of liquid until small traces are left.

11.7 Repeat steps 10.3-10.6 three more times or until sample pH is at least 4.5-7.0. (For some applications it may be necessary to test the amount of glucose present by biochemistry analyser in determining if another wash is necessary. Glucose remaining in the last wash should not exceed more that 0.5 g/L.)

11.8 Record weight of *bottle, cap and sample* to the nearest 0.01g. Then subtract weight of bottle and cap from this recorded weight to caluculate and record the *weight of washed*

sample.

11.9 Perform total solids on the as received and washed material as described in Lap-001.

11.10 Store remaining sample in refrigerator or freezer as appropriate to maintain sample integrity.

12. Procedure B *(Filtration)*

12.1 Record weight of buchner funnel filter cup with glass filter paper and record to the nearest 0.01g on analytical balance and tare.

12.2 Add 25-50 grams of sample. (Record *weight of sample as received*).

12.3 Gradually pour 200-250 ml of deionized water. Stir gently to mix sample. Allow vacuum filtration to proceed slowly.

12.4 Use pH sticks to test the pH of the filtrate and sample.Repeat steps 12.2-12.3 three more times or until sample pH is at least 4.5-7.0. (For some applications it may be necessary to test the amount of glucose present by biochemistry analyser in determining if another wash is necessary. Glucose remaining in the last wash should not exceed more than 0.5 g/L.)

12.5 Let process sit under vacuum for 5 minutes to remove excess water. Remove filter cup and place on paper towel to allow water accumulation on filter cup bottom to escape.

12.6 Weigh filter cup with glass fiber filter paper and sample. Record weight. Subtract the weight of the filter cup and filter to get the *wet weight of the washed sample*. Then place in 105°C oven for overnight.. *(If alternate Buchner funnel is used check with manufactuer for temperature limitations. Use 45°C as oven temperature if necessary and dry to a constant weight.)*

12.7 Remove filter cup with sample from oven and place in desiccator for five minutes. Weigh filter cup + filter + sample and record. Subtract weight of filter cup and filter paper to get dry sample weight. Record *dry weight of washed sample* to the nearest 0.01 grams.

12.8 Perform total solids on the as received material as described in Lap-001. The % total

solids on the washed sample is calculated from *dry weight of washed sample* in step 12.7.

12.9 Discard or store sample as necessary.

13. Calculations

13.1 Calculate the percent insoluble solids and fraction insoluble solids for each sample on a percent dry weight basis. Total solids by LAP-001, on the as received and the washed sample will be necessary for each procedure. However, in *Procedure B (Filtration)* the percent total solids of the washed material is calculated based on the whole sample weight.

Procedure A (Centrifugation)

$$\textit{Dry Weight of Washed sample} = \frac{\textit{(Weight of washed sample) (\% Total solids of washed sample)}}{100}$$

$$\textbf{\textit{\% Insoluble Solids}} = \frac{\textit{Dry weight of washed sample}}{\textit{Weight of sample as received}} \; X\ 100$$

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Weight of washed sample = (Weight of filter cup, filter, & washed sample) – (Weight of filter cup & filter)

***% Total Solids on As Received Sample** = As described in LAP 001*

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